REMARKS

The present Amendment and the following Remarks are submitted in response to the Office Communication mailed June 13, 2005. Applicant thanks the Examiner for entering the previous amendment to the claims, for reviewing the information disclosure statements and for accepting the drawing.

The Examiner was not persuaded by Applicant's remarks in traversing the Restriction Requirement and made the requirement Final. Applicant has amended the claims to express the claimed subject matter in terms of nucleotide sequences encoding a polypeptide with an amino acid sequence of SEQ ID NO:5.

Claims 23-25 and 35-39 have been amended. New claims 41-45 have been added. Claim 40 is canceled. Support for the amendment to claim 24 can be found in the specification at, for example page 10, lines 24-30, page 20, line 31-page 21, line 3 and page 59, lines 5-11. Claims 23-39 and 41-45 are pending upon entry of these amendments.

The Specification has been amended to remove blank spaces and to replace browser-executable code with information identifying the reference source as derived from the website address.

No new matter has been added. The Objections and Rejections raised by the Examiner in the Communication are addressed below.

Objection to the Specification

The specification was objected to because of the presence of browser-executable code and because of missing data corresponding to ATCC accession information. Applicant has amended paragraphs containing this code to cite source information and to disable the code. Applicant also has deleted text referring to ATCC deposit information. In view of these amendments, Applicant requests that these objections be withdrawn.

Rejection of the Claims Under 35 U.S.C. §101

Claims 23-40 were rejected under 35 U.S.C. §101, as allegedly lacking a specific and substantial asserted utility or a well-established utility. In particular, the Examiner states that the disclosure of LGR6 and its tissue distribution fails to provide sufficient evidence on the specific biological functions or physiological significance and thus fails to disclose a patentable utility of the invention. The Examiner asserts that homology of LGR6 with members of the GPCR family does not render the claimed invention a specific and substantial utility. The Examiner further warns against potential propagation of errors in assigning function from predictions based on sequence analysis. The Examiner asserts that the uses disclosed in the specification, such as screening assays, are research uses designed to identify a function of the molecules and are not a substantial utility. The Examiner discounts the disclosure of LGR6 use in

the diagnosis and treatment of weight, cardiovascular, neural or endocrine disorder by saying that they are not specific and substantial because they do not identify or reasonably confirm a real world context of use. The Examiner alleges that the specification does not identify a specific disease and that further research is required to support a specific and substantial asserted utility or a well-established utility. Applicant respectfully traverses this rejection.

Applicant reminds the Examiner that LGR6 is identified as a particular type of GPCR, having an N-terminal extracellular domain comprising leucine rich repeats, in addition to the standard seven transmembrane portion typical of GPCRs. As noted in the specification, at page 17, line27 through page 18, line 25, LGR6 is a member of GPCR subfamily I, which includes the beta2 adrenergic receptor. LGR6 was further identified in a subgroup which includes glycoprotein hormone receptors, which activate the Gs-cAMP signal transduction pathway. In particular, within this subgroup, LGR6 shares highest homology to LGR4 and LGR5. The specification, at page 31, lines 1-8, discloses that LGR6 would signal through cyclic AMP metabolism or phosphatidylinositol turnover. At page 40, lines 1-26, the specification discloses expression patterns for LGR6, with the highest expression in the heart and discloses cardiovascular disorders, including cardiomyopathies and congestive heart failure, where LGR6 would play a role.

Applicant submits Exhibits A and B, which evidence the importance of beta-adrenergic receptors and signaling pathways, including the cAMP pathway, in the development of cardiovascular diseases, such as cardiomyopathy, which lead to heart failure. Exhibits B and C evidence the heterogeneous contributions to heart failure and the need to devise varied strategies to treat this condition. Exhibits D and E evidence that at the time of filing the application to which the present application claims priority, the roles of beta adrenergic receptors and cAMP signaling in heart failure were known to one skilled in the art. As LGR6 is expressed in the heart, is related to the receptors known for roles in heart failure and signals through pathways implicated in heart failure, LGR6 has utility in cardiovascular disorders, as disclosed in the specification at page 40, lines 16-21.

Applicant submits Exhibits F and G as evidence of the expectation that LGR6 plays an important functional role in tissues where it is expressed. LGR4 and LGR5 are the closest relatives to LGR6. Studies wherein the LGR4 or LGR5 gene is deleted show that homozygous mice containing such a deletion have very low, or no survival, respectively. The phenotypic hallmark of such mice is a defect in the tissue in which the LGR is normally expressed. With the highest LGR6 expression in the heart, the heart is most affected by the activity or expression of LGR6. Thus LGR6 is an important target for diagnosis or treatment of cardiovascular disorders.

Applicant asserts that the present application provides a specific, substantial, credible and well-established utility. The utility of using the expression or activity of LGR6 in the diagnosis or treatment of cardiovascular disorders is specific, because a particular set of disorders is disclosed. This utility is

substantial because cardiovascular disorders are a known problem and Exhibit evidence presented herein show that the use of new targets, such as LGR6, is still needed in the "real world" for treatment and diagnosis of these heterogeneous disorders. The utility is credible because at the time of priority date for the application, one of skill in the art would believe the utility of LGR6 for cardiovascular disease, based on the assertions in relation to other known contributors of the disease. This utility is well-established because GPCRs, e.g., of subfamily I and signal transduction, e.g. via cAMP, have well-established roles in disease. In conclusion, Applicants respectfully submit the utility requirement has been met, and the claimed invention is in fact supported by a well established and an asserted specific, substantial utility which is credible. Reconsideration and withdrawal of the rejection under 35 USC 101 is requested.

Rejections of the Claims Under 35 USC §112, First Paragraph

Claims 23-40 are rejected under 35 USC §112, first paragraph. Specifically, the Examiner contends, since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention. Applicants respectfully traverse the rejection. For the reasons stated above, Applicants assert the claimed invention is in fact supported by a specific and substantial utility, and the rejection under 35 USC §112 should be withdrawn.

In case one still needs additional information on how to use the claimed invention for the disclosed utility, Applicant herein highlights some of that information. For example, a utility disclosed in the application is the use of the expression of LGR6 in the treatment or diagnosis of cardiovascular disorders. The detection of nucleic acids is well known to one skilled in the art, and is supplemented by the specification at page 44, line 22 to page 45, line 3; page 46, lines 20-27; page 98, line19 through page 102, line 25 and Example 2, pages 121-122.

For treatment of cardiovascular disorders, antibodies directed to LGR6 polypeptides and antisense molecules directed at LGR6 nucleic acids are described in the specification at pages 65-69 and 52-55, respectively. Methods to administer therapeutic agents directed at modulating the expression or activity of LGR6 are described on pages 108-109. The use of LGR6 in assays to identify modulators of its activity is described at pages 85-93. LGR6 ligands, disclosed in the specification, at page 9, line 6, as glycoprotein hormones (glycoprotein hormones are further illustrated at page 18, lines 15-18) can be useful in the screening assays. Those skilled in the art also know that LGR6 can be assayed even without a ligand, as shown in Exhibits H and I, which describe in vitro assays using orphan GPCRs. These articles show that even at the time of filing the invention, it was recognized in the art that in order to perform the screening assays on GPCRs, including LGR6, one didn't need to have physiological parameters associated with a particular receptor to screen for agonists and antagonists of their function. One only needed to be able to express the receptor in a cell on which a signaling parameter could be

measured. Libraries of compounds, such as those disclosed in the specification on page 87, lines 2-23 could be used to modulate activity from orphan GPCRs expressed and assayed by the methods disclosed in the Exhibits.

As can be seen by these citations to the specification, Applicant has provided much information and many methods to supplement the skill in the art and enable one skilled in the art to make and use the invention without undue experimentation. In view of these remarks, Applicant respectfully requests withdrawal of this rejection.

Claims 38-40 were rejected under 35 USC §112, first paragraph (twice, on pages 8-10 and pages 10-12) for having no structural limitation for the compounds. Applicant is amending claims 38-39 and is canceling claims 40. These claims have definite structure, as defined by the claims on which they depend, and they no longer recite hybridization characteristics of the structure. In view of these amendments, reconsideration and withdrawal of the rejection is respectfully requested.

Rejections of the Claims Under 35 USC §112, Second Paragraph

Claims 38-40 are rejected under 35 USC §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the term "compound" was noted for its general meaning, but its context seemed limited to nucleic acids. Applicant is amending claims 38 and 39 (and canceling claim 40) to delete the term "compound" and leave only the term, "nucleic acid molecule" in the claims. In view of these amendments, Applicant respectfully requests that this rejection be withdrawn.

Rejections of the Claims Under 35 USC §102

Claims 38-40 are rejected under 35 USC §102, as being anticipated by Hillier et al.. Hillier et al. teach a nucleic acid sequence which comprises 512 consecutive nucleotides of SEQ ID NO:4. Applicant is amending claims 38 and 39 (and canceling claim 40) to define the material in the kit in terms of nucleic acid molecules which are much longer than the sequence described in Hillier et al.. In view of these amendments, Applicant respectfully requests that this rejection be withdrawn.

CONCLUSION

The foregoing amendments and remarks are being made to place the Application in condition for allowance. Applicant respectfully requests the timely allowance of the pending claims because, in view of these amendments and remarks, Applicant respectfully submits that the objections to the specification and rejections of the claims under 35 U.S.C. §§ 101, 112 and 102 are overcome. Applicant believes that this application is now in condition for allowance. Early notice to this effect is solicited.

If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is encouraged to call the undersigned. If the Examiner disapproves of Applicant's amendments and remarks in this response, Applicant requests a prompt mailing of a notice to that effect.

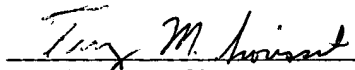
This paper is being filed timely, as a request for a three-month extension of time is being filed concurrently herewith. It is believed no other fees are required. In the event any additional fees, the undersigned hereby authorizes the requisite fees to be charged to Deposit Account No. 501668.

Entry of the remarks made herein is respectfully requested.

Respectfully submitted,

December 13, 2005

MILLENNIUM PHARMACEUTICALS, INC.

By 

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Docket No. MPI99-037P1RCP1CN1M

U.S. Serial No. 10/664,667, Filed September 18, 2003

Exhibits A - I Accompanying Amendment and Response to Office Action Dated June 13, 2005

Exhibit A	Abstract for Wallukat, G., "The beta-adrenergic receptors," *Herz.*, Vol. 27, No. 7 (Nov. 2002) pp 683-690
Exhibit B	Abstract for Movsesian, M. A., et al., "Alterations in cAMP-mediated signaling and their role in the pathophysiology of dilated cardiomyopathy," *Curr. Top. Dev. Biol.*, Vol. 68 (2005) pp 25-48
Exhibit C	Abstract for Sharma, M., et al., "A rational approach for the treatment of acute heart failure: current strategies and future options," *Curr. Opin. Cardiol.*, Vol. 19, No. 3 (May 2004) pp 254-263
Exhibit D	Abstract for Eichhorn, E. J., "Restoring function in failing hearts: the effects of beta blockers," *Am. J. Med.*, Vol. 104, No. 2 (Feb. 1998) pp 163-169
Exhibit E	Abstract for Willette, R. N., et al., "In vitro and in vivo characterization of intrinsic sympathomimetic activity in normal and heart failure rats," *J. Pharmacol. Exp. Ther.*, Vol. 289, No. 1 (Apr. 1999) pp 48-53
Exhibit F	Mazerbourg, Sabine, et al., "Leucine-rich repeat-containing, G protein-coupled receptor 4 null mice exhibit intrauterine growth retardation associated with embryonic and perinatal lethality," *Molecular Endocrinology*, Vol. 18, No. 9 (Sept. 2004) pp 2241-2254
Exhibit G	Morita, Hiroki, et al., "Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension," *Molecular and Cellular Biology*, Vol. 24, No. 22 (Nov. 2004) pp 9736-9743
Exhibit H	Stadel, Jeffrey M., et al., "Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery," *TIPS*, Vol. 18 (Nov. 1997) pp 430-437
Exhibit I	Wilson, Shelagh, et al., "Orphan G-protein-coupled receptors: the next generation of drug targets?" *British Journal of Pharmacology*, Vol. 125 (1998) pp 1387-1392

1: Herz. 2002 Nov;27(7):683-90.

The beta-adrenergic receptors.

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BACKGROUND: The beta-adrenergic receptors of the myocardium play an important role in the regulation of heart function. The beta-adrenergic receptors belong to the family of G-protein coupled receptors. Three subtypes have been distinguished (beta1-, beta2-, and beta3-adrenoceptors). The receptors consist of seven membrane-spanning domains, three intra- and three extracellular loops, one extracellular N-terminal domain, and one intracellular C-terminal tail. PATHOPHYSIOLOGY: Stimulation of beta-adrenergic receptors by catecholamines is realized via the beta-adrenoceptor-adenylylcyclase-protein kinase A cascade. The second messenger is the cyclic AMP (cAMP). Stimulation of the cascade caused an accumulation of the second messenger cAMP and activated via the cAMP the cAMP dependent protein kinase A (PKA) The PKA phosphorylated, beside other cell proteins, the beta-adrenergic receptors. A phosphorylation of the beta-adrenergic receptors caused - with exception of the beta3-adrenoceptor - an uncoupling and desensitisation of the receptors. Phosphorylation via the G-protein receptor kinase (GRK or betaARK) also caused uncoupling and reduced the beta-adrenergic responsiveness. The uncoupling of the receptor is the prerequisite for receptor internalisation. In the process of internalisation the receptor shifted from the sarcolemma membrane into cytosolic compartments. Chronic beta-adrenergic stimulation caused a down-regulation of the receptors. During this process of desensitisation the expression of the receptor on mRNA and protein level is reduced. CHANGING OF THE RECEPTORS IN THE FAILING HEART: In patients with dilated cardiomyopathy the beta-adrenergic responsiveness of the myocardium is diminished. It was shown that in these patients the expression of the beta1-adrenergic receptor is reduced on the mRNA and protein level. In these patients the expression of the inhibitory G-protein G(i) is increased. Furthermore, the expression of the G-protein receptor kinase is elevated. This kinase induces the uncoupling of the beta-adrenergic receptors. These alterations of the beta-adrenoceptor signal cascade may be induced by an elevated catecholamine release or by agonist-like autoantibodies directed against the beta1-adrenergic receptor found in patients with dilated cardiomyopathy. Both, permanent stimulation with catecholamines and chronic treatment with agonistic anti-beta1-adrenoceptor

autoantibodies cause a reduction of the expression of the beta1-adrenoceptor on mRNA and protein level in "in vitro" experiments. Moreover, an over-expression of the beta1-adrenoceptor, the stimulatory G(s) protein, and the protein kinase A induce detrimental alterations of the cardiac function and morphology in transgenic animals. These animals developed heart failure accompanied by an increased mortality rate.

Publication Types:

- Review
- Review, Tutorial

PMID: 12439640 [PubMed - indexed for MEDLINE]

1: Curr Top Dev Biol. 2005;68:25-48. Related Articles, Links

ELSEVIER FULL-TEXT ARTICLE

Alterations in cAMP-mediated signaling and their role in the pathophysiology of dilated cardiomyopathy.

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Dilated cardiomyopathy is a disease characterized by enlargement of the chambers of the heart and a decrease in contractility of the heart muscle. The process involves several alterations in proteins involved in cyclic adenosine monophosphate (cAMP) generation that result in a decrease in intracellular cAMP content per unit of adrenergic stimulation in cardiac myocytes. A fundamental question is whether these changes constitute a pathologic mechanism that contributes to chamber enlargement and hypocontractility or a compensatory adaptation that protects the heart from the adverse effects of increased catecholamine stimulation. Clinical studies in humans suggest that the latter effect may be more important. Studies in animal models, however, make the picture more complex: changes in cAMP-mediated signaling can have different effects depending on the specific protein whose expression or function is altered and the setting in which the alteration occurs. It may be that dilated cardiomyopathy represents a collection of different diseases in which alterations in cAMP-mediated signaling have different roles in the pathophysiology of the disease, and, furthermore, that changes in the phosphorylation of individual substrates of cAMP-dependent protein kinase may be either beneficial or harmful. Identifying differences among patients with dilated cardiomyopathy with respect to the role of altered cAMP-mediated signaling in their pathology, and identifying the "good" and "bad" substrates of cAMP-dependent protein kinase, are important areas for further research.

Publication Types:
- Review

PMID: 16124995 [PubMed - indexed for MEDLINE]

1: Curr Opin Cardiol. 2004 May;19(3):254-63.

LWWonline Lippincott Williams & Wilkins

A rational approach for the treatment of acute heart failure: current strategies and future options.

Sharma M, Teerlink JR.

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PURPOSE OF REVIEW: Acute decompensated heart failure represents a major, growing health problem in the developed world. However, until recently, relatively little research has been performed in this field to provide a basis for rational treatment strategies. The purpose of this review is to discuss the current approach and the potential future strategies for treatment of patients with acute decompensated heart failure. RECENT FINDINGS: Recent data have confirmed the heterogeneous nature of patients admitted with acute decompensated heart failure, and the limitations of the current therapeutic regimens with diuretics, intravenous vasodilators (ie, nitroglycerin, nitroprusside), and intravenous inotropes (ie, dobutamine, milrinone). A new vasodilator, nesiritide, has been demonstrated to improve hemodynamics and symptoms at 3 hours compared with nitroglycerin, and has been added to the therapeutic armamentarium in the United States. However, none of these agents has been shown to influence patient outcomes favorably. Given the high readmission rates, morbidity, and mortality of acute decompensated heart failure, other newer approaches, such as antagonists to a number of neurohumoral targets (ie, endothelin [tezosentan], vasopressin [conivaptan, tolvaptan], and adenosine) and non-cAMP-mediated inotropy (ie, levosimendan), are currently under investigation and showing promise. SUMMARY: Acute decompensated heart failure presents a challenging therapeutic problem for clinicians. Although they readily correct the hemodynamic abnormalities, current treatment strategies have significant limitations and have not been shown to improve morbidity or mortality. A number of new agents are under investigation with the goal of improving patient outcomes.

Publication Types:
- Review
- Review, Tutorial

PMID: 15096959 [PubMed - indexed for MEDLINE]

All: 1 Review: 1

1: Am J Med. 1998 Feb;104(2):163-9.

ELSEVIER FULL-TEXT ARTICLE

Restoring function in failing hearts: the effects of beta blockers.

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Until recently, clinical management of congestive heart failure was purely palliative. The drugs used in patients with failing hearts--digoxin, vasodilators, and positive inotropic agents--improved contractility, reversed hemodynamic abnormalities, and enhanced functional status, but they failed to confer a survival benefit. Indeed, the use of inotropic agents often resulted in excess mortality--a paradox explained in part by the pharmacological properties of these agents, which increase production of cAMP, the intracellular messenger for the beta-adrenergic system. The short-term pharmacological benefits of these drugs may be offset by deleterious long-term biological effects on the heart muscle itself. The use of beta-blockers in heart failure is counterintuitive, given that their initial pharmacological effect is to reduce heart rate and contractility in a faltering heart, thus producing an effect diametrically opposed to that of inotropic agents. However, it is becoming more clear that beta-blocker therapy in patients with heart failure not only improves left ventricular function, but may actually reverse pathological remodeling in the heart. Accumulating clinical evidence indicates that these beneficial changes are the result of secondary biological changes in the myocardium rather than a response to the pharmacological effects of the drugs themselves. Mounting evidence suggest that these agents may prolong survival in patients with heart failure, and ongoing clinical trials may soon confirm these preliminary findings.

Publication Types:

- Review
- Review, Tutorial

PMID: 9528736 [PubMed - indexed for MEDLINE]

☐ 1: J Pharmacol Exp Ther. 1999 Apr;289(1):48-53.

Related Articles, Links

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In vitro and in vivo characterization of intrinsic sympathomimetic activity in normal and heart failure rats.

Willette RN, Aiyar N, Yue TL, Mitchell MP, Disa J, Storer BL, Naselsky DP, Stadel JM, Ohlstein EH, Ruffolo RR Jr.

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Clinical studies conducted with carvedilol suggest that beta-adrenoceptor antagonism is an effective therapeutic approach to the treatment of heart failure. However, many beta-adrenoceptor antagonists are weak partial agonists and possess significant intrinsic sympathomimetic activity (ISA), which may be problematic in the treatment of heart failure. In the present study, the ISAs of bucindolol, xamoterol, bisoprolol, and carvedilol were evaluated and compared in normal rats [Sprague-Dawley (SD)], in rats with confirmed heart failure [spontaneously hypertensive heart failure (SHHF)], and in isolated neonatal rat cardiomyocytes. At equieffective beta1-adrenolytic doses, the administration of xamoterol and bucindolol produced a prolonged, equieffective, and dose-related increase in heart rate in both pithed SD rats (ED50 = 5 and 40 microgram/kg, respectively) and SHHF rats (ED50 = 6 and 30 microgram/kg, respectively). The maximum effect of both compounds in SHHF rats was approximately 50% of that observed in SD rats. In contrast, carvedilol and bisoprolol had no significant effect on resting heart rate in the pithed SD or SHHF rat. The maximum increase in heart rate elicited by xamoterol and bucindolol was inhibited by treatment with propranolol, carvedilol, and betaxolol (beta1-adrenoceptor antagonist) but not by ICI 118551 (beta2-adrenoceptor antagonist) in neonatal rat. When the beta-adrenoceptor-mediated cAMP response was examined in cardiomyocytes, an identical partial agonist/antagonist response profile was observed for all compounds, demonstrating a strong correlation with the in vivo results. In contrast, GTP-sensitive ligand binding and tissue adenylate cyclase activity were not sensitive methods for detecting beta-adrenoceptor partial agonist activity in the heart. In summary, xamoterol and bucindolol, but not carvedilol and bisoprolol, exhibited direct beta1-adrenoceptor-mediated ISA in normal and heart failure rats.

PMID: 10086986 [PubMed - indexed for MEDLINE]

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Molecular Endocrinology 18(9):2241–2254

doi: 10.1210/me.2004-0133

Leucine-Rich Repeat-Containing, G Protein-Coupled Receptor 4 Null Mice Exhibit Intrauterine Growth Retardation Associated with Embryonic and Perinatal Lethality

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Division of Reproductive Biology, Department of Obstetrics and Gynecology (S.M., S.S., C.A.K., J.V.Z, K.K., A.J.W.H.) and Departments of Comparative Medicine (D.M.B.), and Biology (L.V.G, H.R., M.T.-L.), Stanford University, Stanford, California 94305

Leucine-rich repeat-containing, G protein-coupled receptors (LGRs) belong to the largest mammalian superfamily of proteins with seven-transmembrane domains. LGRs can be divided into three subgroups based on their unique domain arrangement. Although two subgroups have been found to be receptors for glycoprotein hormones and relaxin-related ligands, respectively, the third LGR subgroup, consisting of LGR4–6, are orphan receptors with unknown physiological roles. To elucidate the functions of this subgroup of LGRs, LGR4 null mice were generated using a secretory trap approach to delete the majority of the LGR4 gene after the insertion of a β-galactosidase reporter gene immediately after exon 1. Tissues expressing LGR4 were analyzed based on histochemical staining of the transgene driven by the endogenous LGR4 promoter. LGR4 was widely expressed in kidney, adrenal gland, stomach, intestine, heart, bone/cartilage, and other tissues. The expression of LGR4 in these tissues was further confirmed by immunohistochemical studies in wild-type animals. Analysis of the viability of 250 newborn animals suggested a skewed inheritance pattern, indicating that only 40% of the expected LGR4 null mice were born. For the LGR4 null mice viable at birth, most of them died within 2 d. Furthermore, the LGR4 null mice showed intrauterine growth retardation as reflected by a 14% decrease in body weight at birth, together with 30% and 40% decreases in kidney and liver weights, respectively. The present findings demonstrate the widespread expression of LGR4, and an essential role of LGR4 for embryonic growth, as well as kidney and liver development. The observed pre- and postnatal lethality of LGR4 null mice illustrates the importance of the LGR4 signaling system for the survival and growth of animals during the perinatal stage. (*Molecular Endocrinology* 18: 2241–2254, 2004)

THE LEUCINE-RICH REPEAT (LRR)-CONTAINING, G protein-coupled receptors (LGRs) designated as LGR4 through LGR8 are structurally similar to receptors for gonadotropins and TSH (1–3). These receptors are characterized by a large N-terminal extracellular domain-containing LRRs and a seven-transmembrane region. Phylogenetic analysis showed that there are three LGR subfamilies: the classic glycoprotein hormone receptors, the second subgroup of LGR4, LGR5, and LGR6; and a third subgroup represented by LGR7 and LGR8 recently found to be the receptors for relaxin family ligands (4–6). These three subgroups of LGRs have an ancient evolutionary origin and existed before the divergence of vertebrates and invertebrates (1, 2, 6). LGR4, also known as GPR48, is a large protein consisting of 18 extracellular LRRs together with a seven-transmembrane region (1, 7). It shares only around 20% sequence identity with the glycoprotein hormone receptors but exhibited around 45% and 35% homology with LGR5 and LGR6, respectively (1, 7). Unlike LGRs in the other two subfamilies, the ligands and functions for the LGR4/5/6 subfamily are unclear. In an attempt to elucidate the physiological roles of this subgroup of orphan LGRs, we performed gene deletion experiments of the prototypic LGR4 gene using a mouse gene deletion model.

Generation of transgenic mice using the secretory-trap approach is ideal for large-scale functional analysis of secreted and membrane-spanning proteins (8, 9). When the trap vector integrates within the intron of

Abbreviations: AST, Aspartate aminotransferase; BUN, blood urea nitrogen; FAM, 5′-6-carboxy-fluorescein; IGFR-I, IGF receptor type I; IRES, internal ribosome entry sequence; IUGR, intrauterine growth retardation; LGR, leucine-rich repeat-containing, G protein-coupled receptor; LRR, leucine-rich repeat; LRRNT, N-terminal LRR; PLAP, placental alkaline phosphatase; SA, splice acceptor; SD, splice donor; TAMRA, 6-carboxy-tetramethyl-rhodamine; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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a target gene, a β-galactosidase fusion protein, including the N terminus of the target gene driven by its endogenous promoter, is produced. These insertions also effectively mutate the trapped gene to create null alleles. Here, we characterized the phenotypes of LGR4 null mice generated by this secretory trap approach to investigate the physiological roles of this receptor. Taking advantage of the expression of the fusion reporter gene controlled by the endogenous LGR4 promoter, we also performed detailed analysis of the tissue expression pattern of LGR4. The LGR4 gene showed a wide tissue distribution, and its disruption was associated with intrauterine growth retardation (IUGR) and perinatal lethality.

RESULTS

Disruption of the LGR4 Gene in Transgenic Mice

The LGR4 gene was mapped to mouse chromosome 2 (10). The mouse genomic sequence of the LGR4

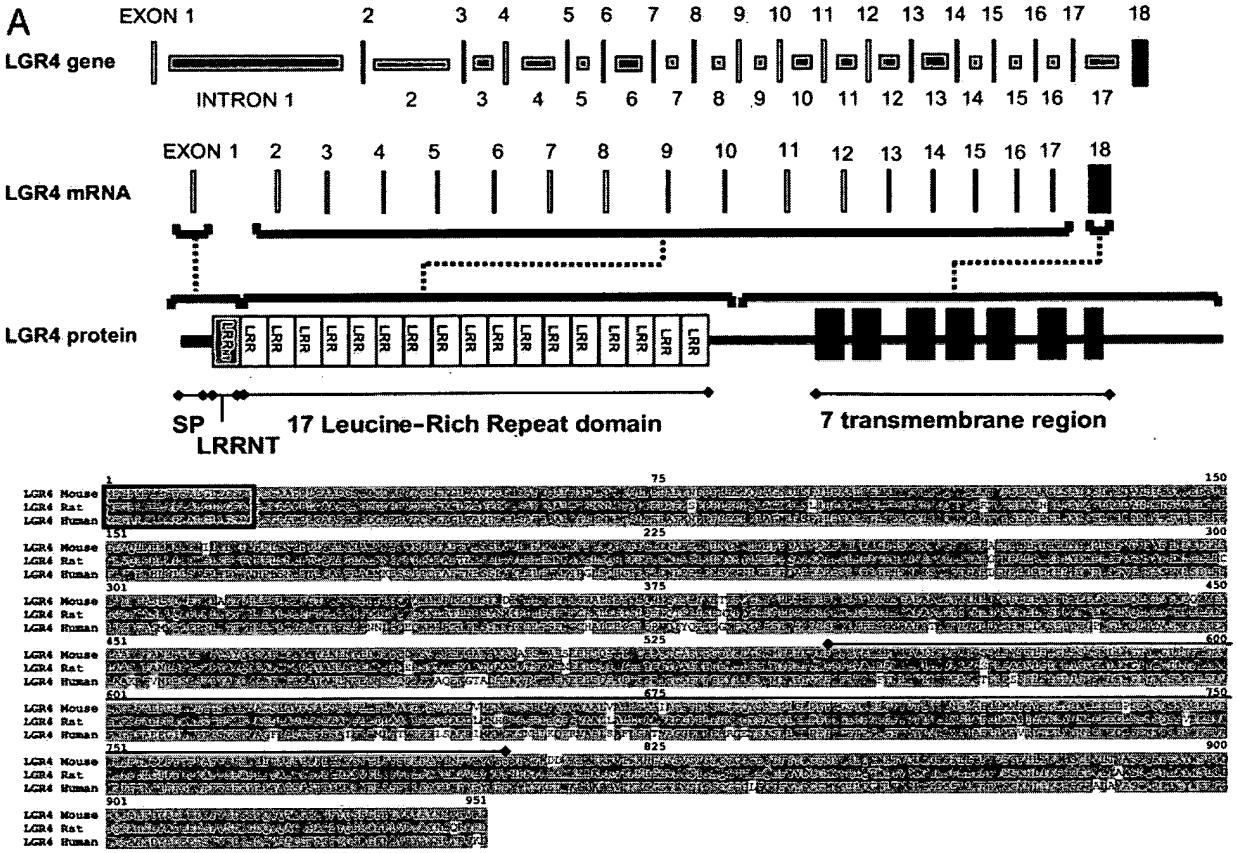

Fig. 1. LGR4 Gene Targeting and Transgene Insertion in Mice as Well as Genotyping Using Genomic DNA

A, Genomic structure and corresponding coding regions of the mouse LGR4 gene. The LGR4 gene contains 18 exons with exon 1 encoding the signal peptide for secretion (SP) and the LRRNT domain. Exons 2–17 encode the 17 LRR domain, and exon 18 encodes the seven transmembrane region plus the C-terminal tail. The *lower panel* shows the alignment of the deduced amino acid sequences of mouse, rat (GenBank accession no. NP_775450) and human (GenBank accession no. NP_060960) LGR4 cDNAs. The full-length cDNA of mouse LGR4 has been deduced from partial cDNA (accession no. AK044357) and genomic (NT_039209) sequences. The predicted signal peptide is *boxed*, the seven-transmembrane domain is *overlined*, and the conserved amino acids are *shaded*. B, Transgene insertion in LGR4 mutant mice. The secretory-trap vector (11.98 kb) includes the mouse En-2 SA sequence, the CD4 transmembrane domain (CD4TM) inserted in frame with β-geo (a fusion gene encoding β-galactosidase, neomycin, and phosphotransferase), followed by the IRES fused to the PLAP gene and the simian virus 40 polyadenylation signal (8, 11, 12). The secretory-trap vector was inserted at the 5′-end of intron 1 of the LGR4 gene. Due to the presence of the SA site in the 5′-end of the insertion vector, transcript splicing occurred between this acceptor site and the SD site in exon 1 of the LGR4 gene. This random insertion resulted in the loss of LGR4 expression and leads to the expression of a chimeric mRNA encoding a protein composed of the LGR4 LRRNT domain and β-galactosidase. In addition, the PLAP protein was also expressed due to the presence of the IRES. Expression of β-galactosidase and PLAP proteins from the trap vector was under the control of the endogenous LGR4 promoter. C, PCR amplification of a LGR4 fragment and the transgene in wild-type (+/+), heterozygous (+/−), and null (−/−) mice. Triplex PCR was performed using genomic DNA as the template together with three primers. Primers A and B allowed the amplification of a LGR4 fragment (A/B, 805 bp) in the wild-type allele whereas primers A and C amplified a transgene fragment (A/C, 650 bp) in the mutant allele. Due to the insertion of the large (11.98 kb) trap vector, primers A and B could not amplify a PCR product in the mutant allele. In heterozygous animals, two PCR products were generated.

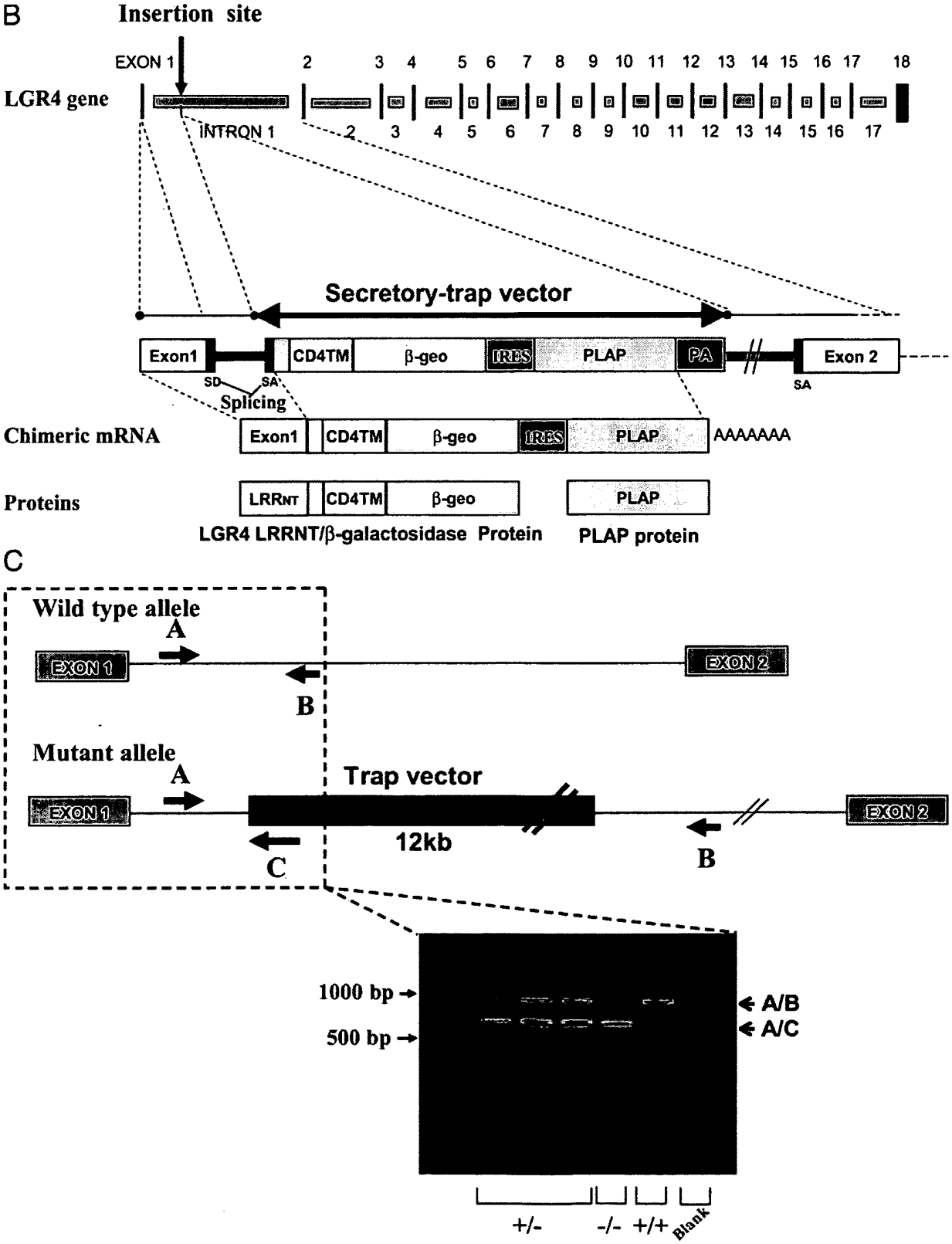

Fig. 1. *Continued*

coding region starts at nucleotide 9579071 and ends at nucleotide 9673612 of the mouse chromosomal contig NT_039209 (GenBank accession number). The predicted full-length cDNA sequence of mouse LGR4 (GenBank accession no. XM_355385) is consistent with the deduced sequence after comparison between the LGR4 partial cDNA (GenBank accession no. AK044357) and the genomic contig (Fig. 1A, *upper panel*). The deduced amino acid sequences of the mouse LGR4 protein show 98% and 95% similarity with the rat (GenBank accession no. NP_775450) and human (GenBank accession no. NP_060960) LGR4 protein sequences, respectively (Fig. 1A, *lower panel*). The mouse LGR4 gene contains 18 exons. Exon 1 encodes the signal peptide (SP) for secretion and the N-terminal LRR domain (LRRNT), whereas exons 2–17 encode the 17 LRR domain, and exon 18 encodes the seven-transmembrane domain as well as the intracellular region (Fig. 1A).

Using the secretory-trap approach, the LGR4 gene was disrupted by random insertion of a secretory-trap vector (Fig. 1B). This vector includes the mouse En-2 splice acceptor sequence (SA), a fragment of CD4 containing the transmembrane domain inserted in frame with βgeo (the LacZ-neomycin-phosphotransferase fusion gene), together with a downstream internal ribosome entry sequence (IRES), the placental alkaline phosphatase (PLAP) gene, and the simian virus 40 polyadenylation signal (8, 11, 12). PCR analyses indicated that the insertion of the secretory-trap vector resulted in the generation of a chimeric transcript containing the first exon of LGR4 fused to the β-geo transcript, suggesting that the splicing occurred between the splice donor (SD) site in exon 1 of the LGR4 gene and the SA site of the trap vector (Fig. 1B).

To delineate the transgene insertion site, we performed PCR by using upstream primers complementary to different regions of intron 1 and a downstream primer complementary to the 5′-end of the trap vector. We localized the insertion site for the secretory-trap vector within intron 1 of the LGR4 gene at 6324 bp upstream from the 3′-end of exon 1 (nucleotide position 9585590 of the chromosome 2 genomic contig sequence, GenBank accession no. NT_039209) (Fig. 1B). By combining three primers (primers A and B, complementary to two sequences in the 5′-region of the LGR4 intron 1 and primer C, complementary to the 5′-end of the trap vector), we demonstrated the amplification by PCR of an expected fusion gene product (A/C; Fig. 1C, *lower panel*) and the wild-type product (A/B; Fig. 1C, *lower panel*) in the heterozygous mice. Only the fusion gene product A/C is present in the homozygous mice. The chimeric transcript was predicted to encode a truncated form of LGR4 that includes its N-terminal leucine-rich repeat (LRRNT) extracellular domain fused to the β-galactosidase (Fig. 1B). The efficient translation of β-galactosidase mRNA allowed convenient analysis of LGR4 expression by staining tissues with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in heterozygous and homozygous animals.

LGR4 Expression Based on β-Galactosidase Transgene Expression in Mutant LGR4 Mice

Whole mount X-gal staining of heterozygous fetuses at embryonic d 19.5 (E19.5) to estimate the β-galactosidase activity showed that the LGR4 gene was widely expressed in diverse tissues (Fig. 2 and Table 1). X-gal staining was observed in brain (periventricular area, PV; olfactory bulb, OB), nasal cavity (NC), spinal column (S) (Fig. 2A, *upper panel*), rib cage (R), heart (H), and intestine (Fig. 2A, *lower panel*). In contrast, the wild-type fetus at E19.5 showed a background staining only in the intestine (Fig. 2B, *arrows*). In the heterozygous fetuses, kidney and adrenal gland (Fig. 2C), as well as stomach, bladder, bone, and cartilage (Table 1), also showed a strong β-galactosidase expression. In contrast, liver, thymus, lung (Fig. 2A, *lower panel*), spleen, pancreas, and skeletal muscle did not show X-gal staining (Table 1). In adult heterozygous mice, whole-mount X-gal staining showed a similar expression pattern in most organs similar to that found in the fetuses (Table 1). However, in the adult, β-galactosidase expression was weaker in the heart but stronger in the liver (Table 1).

To determine the exact cell types expressing the β-galactosidase transgene, X-gal staining was done on selective organ sections of heterozygous and homozygous newborns (day of birth, D0). In kidney sections, the transgene was found in the epithelial cells of some tubules (t) (Fig. 2D, *arrows*) and Bowman's capsule of glomeruli (g) (Fig. 2D, *arrowheads*). The transgene was also expressed in the cardiac muscle of the heart, the epithelium, and the smooth muscle layer of the digestive tract of newborns (data not shown). Although no staining was apparent on the whole organ, low levels of staining were observed in liver, spleen, and medulla of thymus (Table 1).

LGR4 Expression Based on Immunohistochemical Analysis of Endogenous LGR4 in Wild-Type Mice

In addition to studies of transgene expression, we further analyzed endogenous LGR4 expression in wild-type animals by using a specific antibody generated against the ectodomain of LGR4. In the kidney, specific LGR4 staining was found in epithelial cells of selective tubules (t) (Fig. 3A, *arrows*). In contrast, no immunoreactivity was observed in the kidney stained with the preimmune serum (Fig. 3B). A strong immunoreactivity was also present in the zona fasciculata (f), but lower in the zona glomerulosa (g) of the adrenal gland (Fig. 3C). Furthermore, immunoreactive LGR4 was found in the epithelial cells (e) of the gut (Fig. 3D, *arrow*), together with lesser staining in the muscularis mucosae layer (m) (Fig. 3D). In addition, positive staining was found in the keratinized stratified squamous epithelium (e) of the nonglandular stomach (Fig. 3E, *arrow*). Only background immunoreactivity was observed in the stomach stained with the preimmune

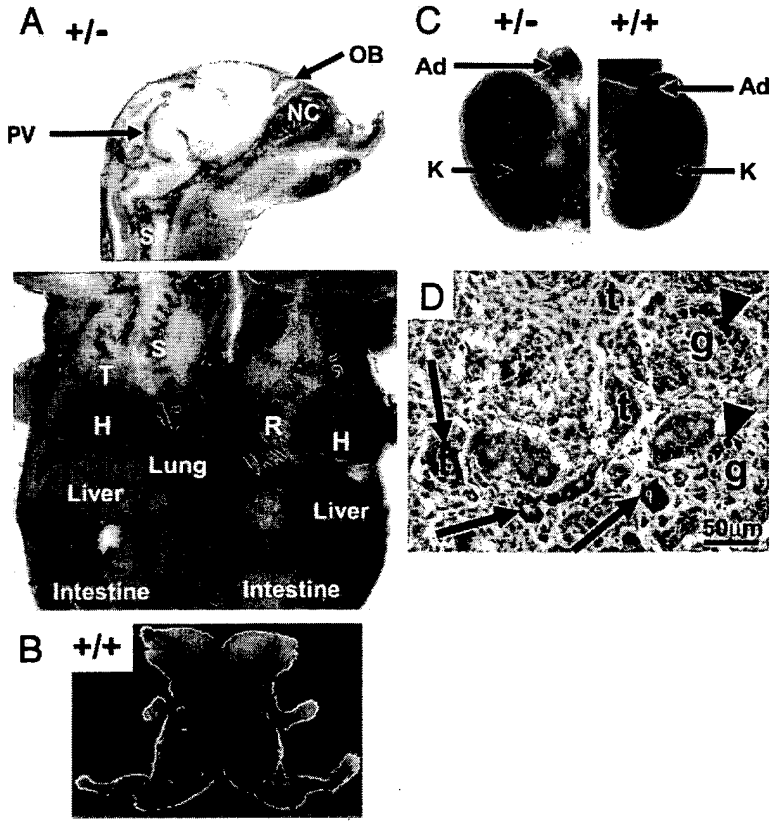

Fig. 2. Diverse Tissue Expression of the β-Galactosidase Reporter Gene Driven by the Endogenous LGR4 Promoter in LGR4 Mutant Mice

A, Whole-mount staining of heterozygous (+/−) animals at embryonic d 19.5 (E19.5). Transverse sections of the embryo showed β-galactosidase activity in different organs. *Upper panel*, Positive staining in the periventricular (PV) area, olfactory bulb (OB), nasal cavity (NC), and spinal column (S). *Lower panel*, Positive staining in the heart (H), intestine, and ribs (R), and minimal staining in the liver. No staining was apparent in the thymus (T) and lung. B, Whole-mount staining of wild-type animals at E19.5 served as a control. C, β-Galactosidase activity in the kidney (K) and adrenal gland (Ad) at embryonic d 19.5 (E19.5) of heterozygous (+/−) but not wild-type embryos (+/+). D, β-Galactosidase staining of kidney sections in null (−/−) newborns indicated strong staining in the parietal epithelium of Bowman's capsule (*arrowheads*) surrounding the glomerulus (g) and in the epithelium of some tubules (t) (*arrows*). Sections were counterstained with Nuclear Fast Red.

serum (Fig. 3F). These data confirmed the wide tissue distribution of the LGR4 receptor observed using the X-gal staining.

Perinatal Lethality of LGR4 Null Mice

We compared the litter sizes among seven intercrosses of wild-type (+/+) parents, 14 intercrosses of heterozygous (+/−) parents, and 14 intercrosses between heterozygous (+/−) and wild-type (+/+) parents. As shown in Fig. 4 (D0), a 17% decrease in litter size was found in the heterozygous intercrosses (8.6 ± 0.5 pups per litter) as compared with those from wild-type parents (10.4 ± 0.5 pups per litter). At weaning (Fig. 4; D21), further decreases in offspring survival were evident in heterozygous intercrosses (+/− × +/−; 6.5 ± 0.6 pups per litter) as compared with wild-type intercrosses (+/+ × +/+; 10 ± 0.4 pups per litter) ($P < 0.05$).

We further genotyped individual pups from 27 heterozygous intercrosses to estimate the ratio of wild-

Table 1. Tissue Expression Pattern for LGR4 in Fetal/Newborn and Adult Mice

	Fetus/Newborn	Adult
Kidney	+	+
Adrenal gland	+	+
Stomach	+	+
Intestine	+	+
Bladder	+	+
Heart	+	+/−
Brain areas	+	+
Bone/cartilage	+	+
Liver	+/−	+
Lung	+/−	−
Spleen	+/−	−
Thymus	+/−	ND
Pancreas	−	−
Skeletic muscle	−	−

Tissues expressing LGR4 were deduced based on the expression of the β-galactosidase transgene using whole-mount (E19.5 and adult mice) and tissue sections (newborn). +, Positive expression; +/−, low expression; −, negligible expression; ND, not determined.

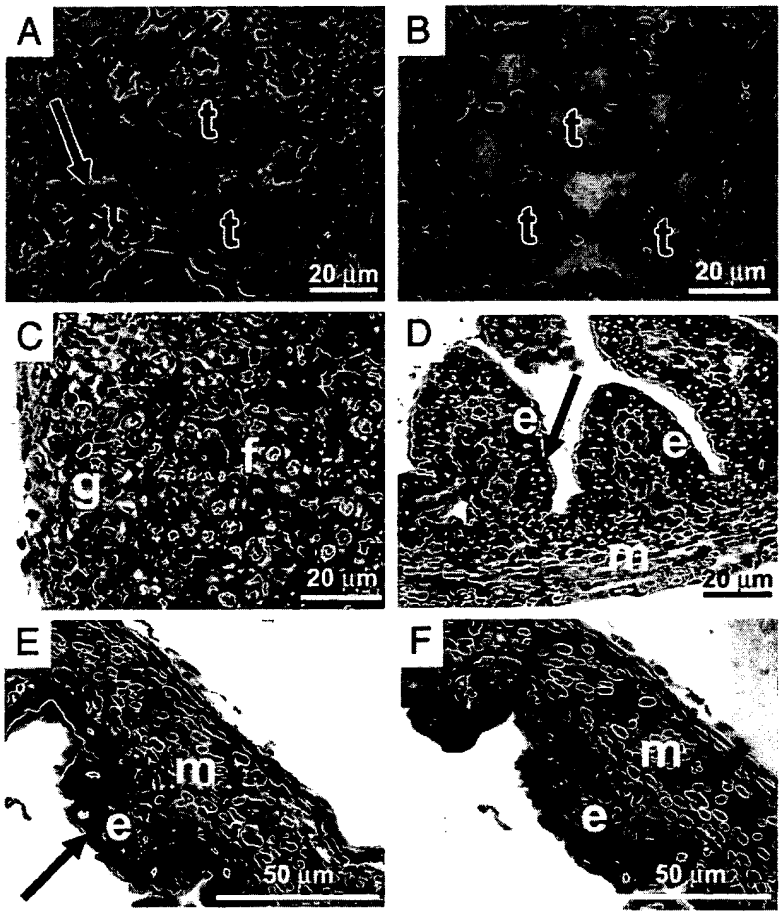

Fig. 3. Immunolocalization of LGR4 in Wild-Type Newborn Mice Using Antibodies against the LGR4 Ectodomain

A, Epithelial cells of selective cortical renal tubules (t) (*arrows*) in the kidney cortex showed positive staining. B, No staining was detected in the section of cortex incubated with nonimmune serum. C, The zona fasciculata (f) of the adrenal gland was immunoreactive in contrast to low staining in the zona glomerulosa (g). D, Intestinal epithelial cells (e) were stained positively (*arrow*), whereas the muscularis mucosae (m) showed minimal reactivity. E, The keratinized, stratified squamous epithelium of the nonglandular stomach (e) (*arrow*) showed positive staining. F, Background staining was detected in a stomach section incubated with nonimmune serum.

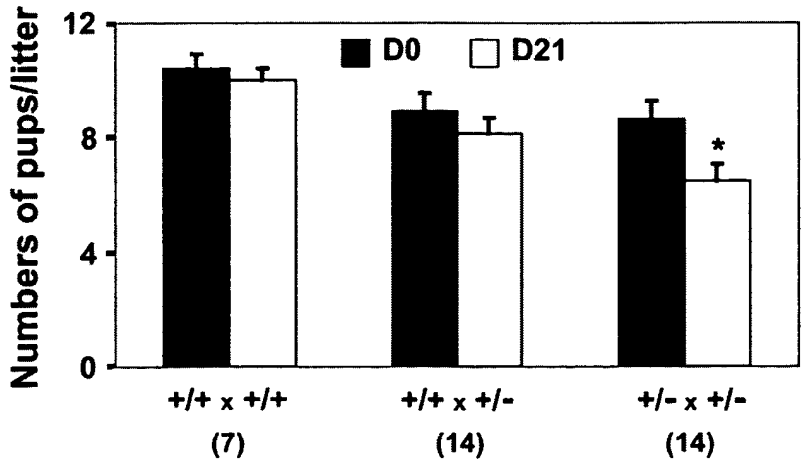

Fig. 4. Litter Size at Birth and d 21 of Age (D21) in Wild-Type and Mutant LGR4 Mice

The number of pups per litter was determined from wild-type (+/+) and heterozygous (+/−) breedings at birth [d 0 (D0) and weaning (D21). *Numbers in parentheses* indicate the number of litters studied. Results are presented as the mean ± SEM. *, $P < 0.05$, −/− *vs.* +/+.

type, heterozygous, and null mice in the offspring. Based on the assumption that all wild-type pups, constituting 25% of offspring, are viable, we observed a decrease in the expected number of heterozygous and homozygous mice at birth (Table 2, *upper portion*). These data suggested that fetal death occurred during embryonic development, and only 40% of the expected homozygous pups were born. These LGR4 null mice never showed any apparent signs of respiratory distress but were smaller in size and became progressively weaker after birth. Indeed, most (eight of 14) of the surviving LGR4 null newborns died postnatally at 1 d after birth (D1). In addition, the heterozygous mice also showed embryonic lethality with 90% of the expected heterozygous pups found at birth, and 19% (14 of 73) of them died postnatally (Table 2, *upper portion*). Only one female and two male LGR4 null mice survived until the adult stage (Table 2, *upper portion*). They did not present obvious differences in external phenotypes in comparison with their littermates. Breeding tests indicated that both male and female LGR4 null mice were infertile. After being killed, the two LGR4 null males showed swollen testes and kidneys whereas the one LGR4 null female exhibited enlarged uteri.

To confirm the embryonic lethality of mutant mice, a group of seven pregnant heterozygous mice were killed for the genotyping of their fetuses at embryonic day 19.5. As shown in Table 2 (*lower panel*), only 76% and 91% of the expected homozygous and heterozygous pups were present, respectively. Furthermore, five of 13 (38.5%) homozygous fetuses at E19.5 were not viable at the time of surgery. These data confirm the predicted embryonic lethality of the LGR4 null fetuses.

Decreases in Body Weight and Lower Kidney and Liver Weights in Newborn LGR4 Null Mice

Although no gross morphological abnormalities were found for the LGR4 null mice, 14% and 20% decreases ($P < 0.05$) in body weight were observed at birth and on day 1, respectively, as compared with their wild-type littermates (Fig. 5A). In contrast, there were no differences between the body weight of heterozygous and wild-type mice. We further analyzed individual organ weights of these animals. The absolute organ weight of the kidney, liver, heart, lung, thymus, and spleen showed significant reduction in the homozygous newborns compared with the heterozygous and wild-type newborns (Fig. 5B). However, when the ratio of organ to body weight was compared, a major reduction of the ratio was found only for kidney and liver in the LGR4 null newborns as compared with the heterozygous and wild-type mice (Fig. 5C).

Histological Analysis of Organs of LGR4 Null Newborns

The observed decreases in kidney weight in LGR4 null newborns was confirmed by histological analysis, showing a size reduction (Fig. 6, A and B). However, the kidneys from LGR4 null mice showed similar his-

Table 2. Neonatal and Embryonic Lethality of LGR4 Null Mice

Neonatal lethality		+/+	+/−	−/−	Total
No. of pups at birth	Observed	77	138	31	246
	Expected	77	154	77	
	Observed/expected	100%	90%	40%	
No. of pups dead after birth/total pups		2/34	14/73	11/14	121[a]
Day of death	D0 (day of birth)		1		
	D1	1	11	8	
	D2	1	2	1	
	D4			1	
	D6			1	
				3 alive	

Embryonic lethality		+/+	+/−	−/−	Total
Number of pups *in utero* at E19.5	Observed (alive/dead)	17/0	31/3	13/5	61/8
	Expected	17	34	17	
	Observed/expected	100%	91%	76%	

Genotyping was performed for intercrosses of heterozygous mice after birth. Based on the predicted ratio of wild-type and mutant animals at birth, embryonic lethality for LGR4 null mice could be inferred because only 40% of the expected homozygous pups were born. Most (eight of 14) of the surviving LGR4 null newborns showed postnatal lethality at 1 d after birth (D1). Although the heterozygous mice showed limited embryonic lethality (90% of the expected heterozygous pups were born), 19% of them died postnatally. Animal viability was recorded only for mice not used for experimental manipulation and after natural birth. To confirm embryonic lethality of mutant mice, genotyping was also performed at E19.5 (*lower portion*). Only 76% of the expected homozygous pups were present. Five of 13 homozygous fetuses were dead *in utero*.

[a] Fewer number of animals studied due to killing at birth to perform histological and serum analysis.

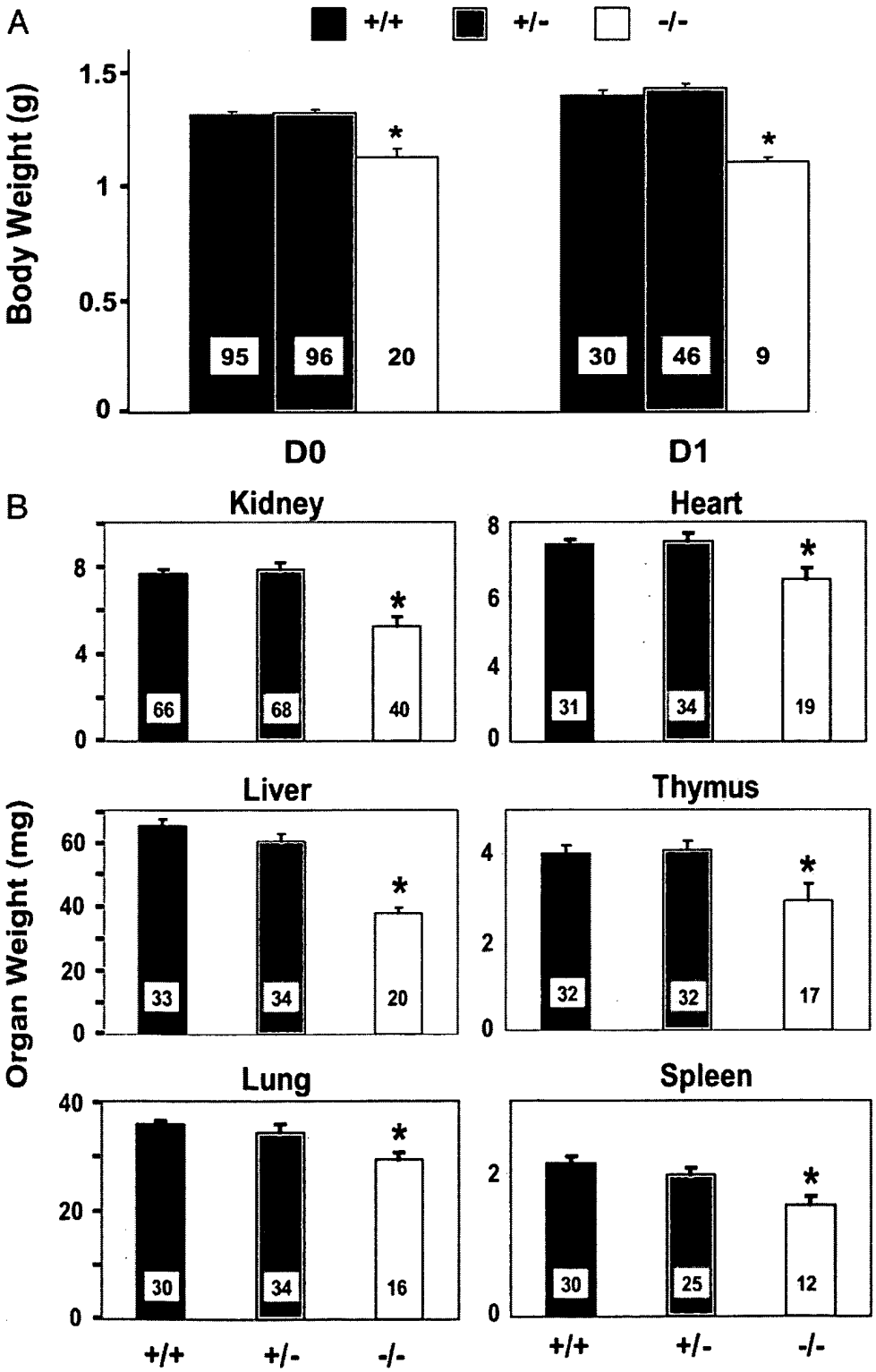

Fig. 5. Body and Organ Weights of Wild-Type and Mutant LGR4 Mice

A, Body weight of newborns [d 0 (D0) and 1-d-old pups (D1)]. B, Absolute organ weights and (C) ratio of organ weight to body weight in wild-type and mutant newborns. *Numbers inside the bars* indicate the number of animals studied. Results are presented as the mean ± SEM; *, $P < 0.05$, $-/-$ *vs.* $+/+$ and $+/-$.

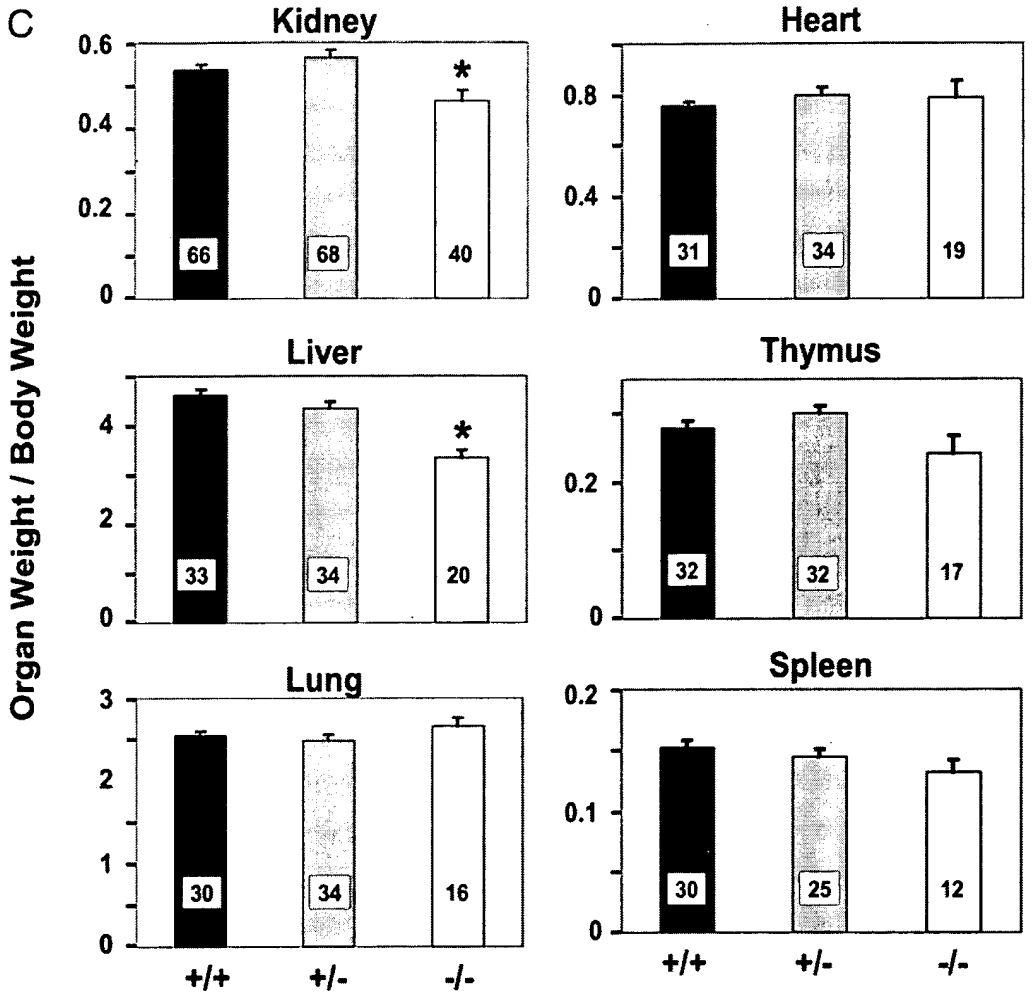

Fig. 5. *Continued*

toarchitecture as compared with their wild-type counterparts (Fig. 6, A–D). No apparent difference in the cortico (co)-medullary (m) proportions and no obvious structural abnormality of the glomeruli (*arrows*) or tubules (*arrowheads*) were observed in the LGR4 null mice (Fig. 6D) as compared with wild-type animals (Fig. 6C).

In addition to the kidney, the liver of LGR4 null newborns also showed a major weight reduction. However, histological analysis did not reveal any deviations from normal structure (data not shown). Similarly, no gross or histological abnormalities were noted in the stomach, intestine, urinary bladder, heart, lung, spleen, thymus, or pancreas (data not shown).

Lack of Changes in the Expression of IGF-I, IGF-II, and IGF Receptor Type-I (IGFR-I) in LGR4 Null Mice

Earlier experiments have demonstrated the importance of the IGF signaling system for embryonic and neonatal body and organ growth (13–16). In particular, liver-derived IGF-I has been shown to be essential for *in utero* body growth (17), and kidney IGF-II is involved in renal development (18, 19). We tested possible changes in the expression levels of IGF-I, IGF-II, and IGFR-I transcripts in liver and kidney between wild-type and null newborn mice by RT-PCR. As shown in Fig. 7, no differences in the relative expression level of IGF-I, IGF-II, and IGFR-I in the liver (*upper panel*) and the kidney (*lower panel*) could be found between wild-type and null newborn mice.

Assays of Serum and Urine Markers

Serum chemistry analyses for blood urea nitrogen (BUN) and creatinine are routinely used to evaluate renal functions (20), and the liver function during perinatal period was assessed by the measurement of aspartate aminotransferase (AST) (21) and serum glucose levels (22). Serum and urine samples were collected at birth from wild-type and LGR4 mutant mice to perform marker tests. As shown in Table 3, no significant differences in serum glucose, AST, and BUN or urine BUN and creatinine levels were found between wild-type, heterozygous, and homozygous newborn mice.

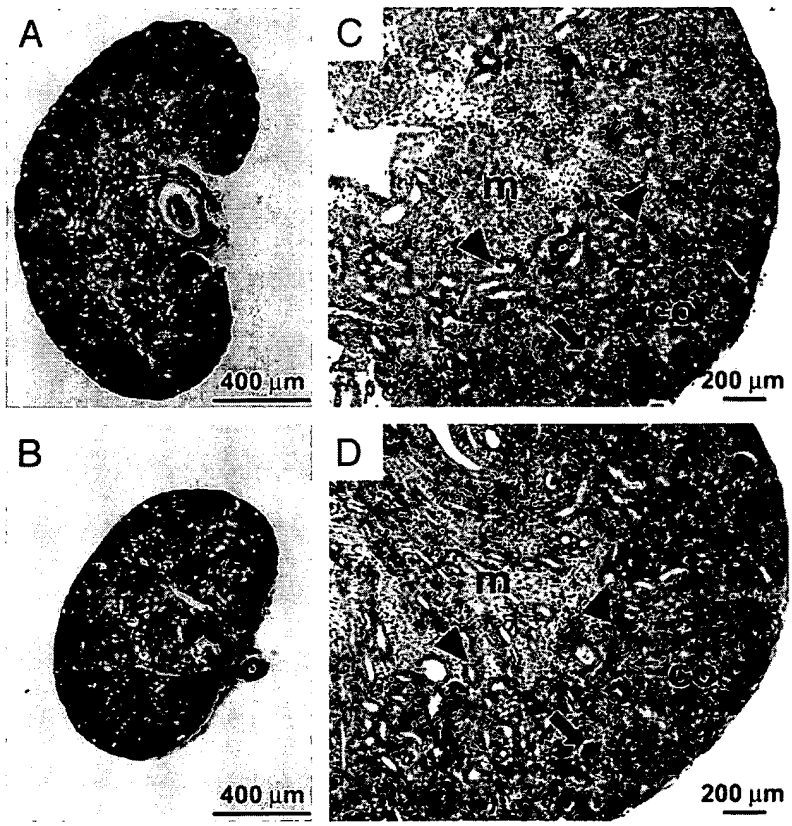

Fig. 6. Histological Analysis of Kidney in Wild-Type and LGR4 Null Newborn Mice

Hematoxylin-eosin-stained sagittal section of the kidney from the LGR4 null newborn (B) demonstrates the smaller compared with its wild-type littermate (A). Higher magnification of a kidney section shows the cortex (co) and the medulla (m), which contain glomeruli (*arrows*) and tubules (*arrowheads*). Other than size, no obvious differences were observed between LGR4 null (D) and wild-type (C) animals.

DISCUSSION

LGR4 is an orphan receptor belonging to the LGR superfamily. Here, we characterized the expression pattern of LGR4 and investigated its physiological roles in null mice generated by a gene trap approach. Histochemical staining of endogenous LGR4 protein and β-galactosidase transgene driven by the LGR4 gene promoter showed that LGR4 is highly expressed in kidney, adrenal gland, stomach, intestine, cartilage, bone, and heart of newborn mice. Genotyping and animal survival analysis of heterozygous intercrosses revealed embryonic lethality of the LGR4 homozygous fetuses. For the LGR4 null mice that survived at birth, most of them died within the first 2 d of life. Furthermore, they showed a 14% decrease in body weight at birth, together with decreases in kidney and liver weights of 30% and 40%, respectively. Histological analyses, however, did not reveal any kidney or liver abnormalities. The observed pre- and postnatal lethality, as well as the IUGR of LGR4 null mice, underlies the importance of the LGR4 signaling system for the survival and the growth of animals during the perinatal stage.

LGR4 null mice were generated using the secretory trap approach (9, 11, 12). The trapping of the LGR4 gene generated a fusion protein consisting of a portion of the extracellular domain of the LGR4 receptor fused to an intracellular membrane-tethered β-galactosidase enzyme. The fusion proteins were sequestrated in the endoplasmic reticulum, minimizing any effect they may have at the cell surface (8). Creation of the LGR4 null mice was demonstrated by the PCR amplification of a fusion product between the LGR4 intron 1 and the trap vector, the absence of LGR4 wild-type transcripts, and the detection of the β-galactosidase activity.

Taking advantage of the expression of the β-galactosidase reporter gene driven by the endogenous LGR4 promoter, the expression of LGR4 was studied in heterozygous and homozygous mice (11). X-gal staining and PCR analysis (data not shown) of tissues from transgenic fetuses and newborns showed that the LGR4 gene was highly expressed in diverse tissues. Immunohistochemical staining of LGR4 of wild-type tissues further confirmed the LGR4 gene expression pattern, with high levels in epithelial cells of the kidney tubules and the gut, in the cardiac muscle, and in the zona fasciculata of the adrenal gland. A similar tissue expression profile was found in the adult, with the exception of higher expression of LGR4 in the liver and lower expression in the heart. These data are consistent with earlier analysis of

LGR4 mRNA expression based on Northern blotting (7). Using the transgene expression analysis, we did not confirm the high expression of LGR4 mRNA in muscle observed by Loh *et al.* (7). The nature of this apparent discrepancy is not clear. Although similar patterns of LGR4 mRNA expression were found for most human tissues, human pancreas showed the highest transcript level (1, 7), perhaps due to species variations. Nevertheless, overall results suggest a wide tissue expression of LGR4. In addition to its expression in adults, LGR4 is also expressed in embryos (7) and could be involved in embryonic development.

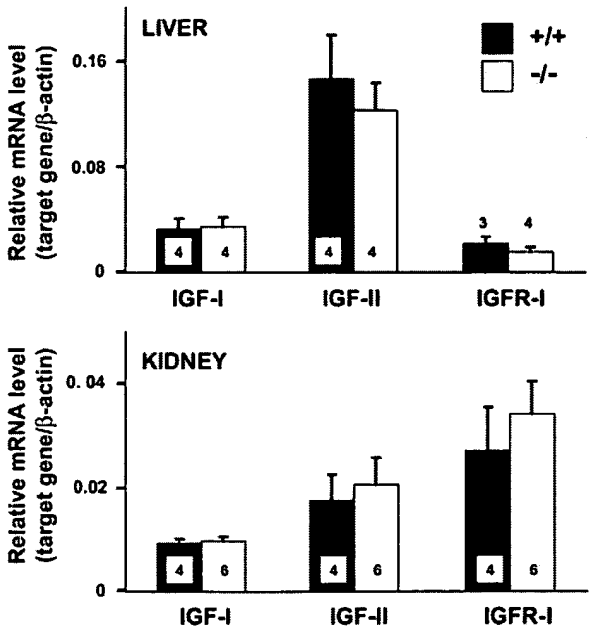

Fig. 7. IGF-I, IGF-II, and IGFR-I Transcript Levels in Liver and Kidney of Wild-Type and LGR4 Null Newborn Mice

Real-time PCR was performed to estimate the mRNA levels for IGF-I and related genes in liver (*top panel*) and kidney (*lower panel*). β-Actin transcript levels were also estimated for all samples to derive the target gene/β-actin ratios. *Numbers inside the bars* indicate the number of animals studied. Results are presented as the mean ± SEM.

LGR4 null mice showed a 14% reduction in body weight at birth, associated with pronounced decreases of kidney and liver weights. Similar weight reductions were observed in newborns of humans, rodents, and pigs exhibiting the syndrome of IUGR (23–27). Although IUGR has been associated with intrauterine infections, genetic factors, uteroplacental deficiency, and diseases of the mother, the causes for 40% of these cases are still unknown (28). Defects in the IGF and insulin pathways constitute examples of hormonal signaling abnormalities associated with IUGR (29, 30). The present finding of severe IUGR phenotypes in LGR4 null mice suggested that the LGR4 receptor pathway could be important in fetal growth.

Similar to LGR4 null mice, low body weight at birth has been found in mice with the mutated IGF-I, IGF-II, and IGFR-I gene (14, 15, 31, 32). Tissue-specific deletion of IGF-I in the liver further revealed that hepatic-derived IGF-I was necessary for intrauterine growth (17). However, in LGR4 null mice, hepatic IGF-I, IGF-II, and IGFR-I transcript levels were not changed, suggesting circulating levels of IGFs were maintained in LGR4 null newborns. Furthermore, LGR4 null mice did not show impaired development of the diaphragm and intercostal muscles found in IGF-I null mice (15). Moreover, an increase in liver weight was found in IGF-I null mice (14), whereas a decrease in liver weight was observed in LGR4 null mice. Apart from the common growth retardation found in LGR4 and IGF-II null newborns, IGF-II null mice did not exhibit perinatal lethality (31). These data suggest that the severe defects of LGR4 deficiency cannot be explained by decreases in IGF-II expression during embryonic development.

Although IUGR is usually associated with increases in perinatal mortality, the LGR4 null mice showed a more severe phenotype. Most of the LGR4 null newborn mice (eight of 14) and some heterozygotes (11 of 73) died on the first day after birth. However, these pups breathed normally, and milk was found in their stomachs. They became progressively weaker and eventually showed postnatal lethality due to unknown causes. The observed lethality of heterozygous LGR4 pups suggests the importance of the LGR4 gene in newborn survival. The essential role of LGR4 in overall

Table 3. Serum and Urine Markers in Wild-Type and Mutant LGR4 Mice

	+/+	+/−	−/−
Serum			
Glucose (mg/dl)	35 ± 6 (3)	21 ± 8 (3)	23 ± 6 (7)
AST (IU/liter)	156 ± 13 (6)	171 ± 22 (7)	200 ± 22 (12)
BUN (mg/dl)	22 ± 2 (10)	24 ± 3 (8)	20 ± 6 (3)
Urine			
BUN (mg/dl)	89 ± 12 (7)	79 ± 12 (16)	153 ± 41 (4)
Creatinine (mg/dl)	4 ± 1 (7)	4 ± 1 (16)	6 ± 1 (4)
BUN/creatinine	25 ± 3 (7)	22 ± 3 (10)	24.5 ± 1 (4)

Serum and urine samples were collected on the day of birth and used to measure various markers for kidney and liver functions. Mean ± SEM. *Numbers in parentheses* indicate the number of animals analyzed. No statistically significant differences were found between different groups.

development was underscored by intrauterine death, evidenced by a reduced number of homozygous and heterozygous pups at birth and by the presence of nonviable fetuses *in utero*.

In LGR4 null mice, the kidney showed a 30% weight reduction compared with wild-type animals. This phenotype could be the consequence of defects in renal morphogenesis, which is dependent upon the IGF signaling system (33). IGF-I, IGF-II, and IGFR-I are expressed in the mouse fetal kidney and IGF-II is the major fetal form essential for kidney differentiation (19) and metanephros growth (18). However, in the kidneys of LGR4 null newborns, we did not observe a decrease in IGF-I, IGF-II, and IGFR-I mRNA levels, suggesting that the IGF paracrine system was not disrupted at this stage of development. Indeed, histological analysis of kidney sections did not show any structural abnormalities. Using platelet-endothelial cell adhesion molecule-1 immunostaining of the endothelial cells of the glomeruli capillaries (34), we did not observe obvious abnormalities in the vascularity of the glomeruli in LGR4 null newborn kidneys (data not shown). Likewise, serum BUN levels were similar in wild-type, heterozygous, and homozygous newborns. These data suggest that the kidney filtration ability was not altered in LGR4 null mice, and kidney malfunction does not likely account for the perinatal death of the newborns.

LGR4 gene disruption affected liver growth with a 40% weight reduction. However, we did not observe hepatic structural abnormalities in LGR4 null mice. Disproportionate alteration of the fetal liver mass has been described in disorders that cause augmented or diminished somatic growth. In the rat, IUGR induced by artery ligation or maternal fasting is associated with a 30% decrease in liver weight (23, 35). However, our data showed that LGR4 expression was minimal in newborn liver, suggesting that the observed liver abnormality could be due to LGR4 action in extrahepatic sites. Despite the observed reduction in liver size in LGR4 null mice, we did not observe impaired liver function. Serum levels of the hepatic enzyme, AST (21), were similar between wild-type and null newborns. We also did not detect significant changes in serum glucose levels. Glucose is a major metabolic fuel during perinatal development, and failure to correct postnatal hypoglycemia is lethal in neonatal rats (22). Normal serum glucose levels observed in LGR4 null newborn suggest that liver glycogenolysis and gluconeogenesis were not altered (22, 36).

The present results demonstrate the wide tissue expression of LGR4 and the importance of this orphan receptor in embryonic and neonatal development. The observed decreases in body growth and organ development suggest an IUGR phenotype. Both fetal and perinatal mortality occurred, not allowing the identification of a single cause of death. Future development of tissue-specific LGR4 null mice is needed to elucidate the essential role of LGR4 in kidney and diverse other tissues expressing this orphan receptor. In addition, the eventual identification of the cognate ligand for this receptor could further advance our understanding of the physiological roles of this ligand/receptor system during fetal and neonatal development.

MATERIALS AND METHODS

Generation of Transgenic Mice by the Gene-Trap Strategy

LGR4 null mice were generated based on the secretory-trap approach as previously described (8, 11, 12) by disrupting the endogenous LGR4 gene (Fig. 1B). The secretory-trap vector (pGT0,1,2tm-pfs, 11.98 kb) includes the mouse En-2 SA sequence, a fragment of CD4 containing the transmembrane domain inserted in frame with βgeo (the LacZ-neomycin-phosphotransferase fusion gene) together with a downstream IRES, the PLAP gene, and the simian virus 40 polyadenylation signal in the pGT1tm vector (Fig. 1B) (8, 11, 12). PCR analyses indicated that transcript splicing occurred between the splice donor site in exon 1 of the LGR4 gene and the SA site of the trap vector. This insertion resulted in the generation of two proteins. The first chimeric protein composed of the first 151 amino acids of LGR4 corresponding to the LRRNT type domain fused to the transmembrane region of CD4, together with the β-galactosidase enzyme encoded by the trap vector. Due to the presence of the IRES, the PLAP protein was also generated.

Mouse Husbandry and Genotyping

Mice were housed under controlled humidity, temperature, and light regimen and fed *ad libitum*. Animal care was consistent with institutional and NIH guidelines. Heterozygous C57Bl/6J mice were intercrossed with Swiss Webster mice; 35 intercross litters were obtained by pairing male and female animals overnight. The day when the copulation plug was observed was considered as embryonic d 0.5 (E0.5) whereas the day of birth was designated as d 0 (D0). Approximately 250 mice (27 litters) from heterozygous intercrosses were genotyped to perform the present study. For genotyping, genomic DNA was isolated from tail biopsy specimens using the genomic DNA extraction kit (Promega Corp., Madison, WI). PCR was carried out using three primers (Fig. 1C): upstream primer A, 5′-CCAGTCACCACTCTTACACAATGGCTAAC-3′ (nucleotide 9585044 to 9585072 in the mouse chromosome 2 contig) located in intron 1 of LGR4; downstream primer B, 5′-ATTCCCGTAGGAGATAGCGTCCTAG-3′ (nucleotide 9586038 to 9586062) in intron 1 of LGR4; and the second downstream primer C, 5′-GGTCTTTGAGCACCAGAGGAC-3′ (5′-end of the trap vector). Two PCR products were expected: the wild-type PCR product A/B (805 kb) and the transgene PCR product A/C (650 kb). Due to the insertion of the large 11.98-kb trap vector between primer A and primer B, the A/B PCR product could not be amplified in the transgene allele under the present PCR conditions (Fig. 1C).

X-gal Staining and Histological Analyses

To perform X-gal staining in heterozygous and homozygous newborns, whole embryos (E19.5) or organs were dissected in PBS and incubated with the β-galactosidase fixative (0.2% glutaraldehyde, 1.5% formaldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.1 M sodium phosphate buffer, pH 8) for 15 min to 5 h. Skin was removed from embryos before fixation. Tissues were washed three times for 30 min in the washing buffer [0.1 M sodium phosphate buffer (pH 8), 2 mM $MgCl_2$, 5 mM EGTA, 0.01% (wt/vol) sodium deoxycholate, 0.02% (vol/vol) Nonidet

P40]. Subsequently, tissues were incubated in the staining solution [washing buffer containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6{\cdot}6\ H_2O$, and 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Invitrogen, Carlsbad, CA)] for 3 h at room temperature. The tissues were then rinsed in PBS before fixation in 3.7% formaldehyde overnight at 4 C. Photography was performed with transillumination using a dissecting microscope (Leica Microsystem, Bannockburn, IL). Wild-type newborns were used as negative controls. To perform β-galactosidase staining of tissue sections, organs were embedded in Tissue-Tek (Sakura Finetek, Torrance, CA), frozen on dry ice, and stored at −80 C. Cryosections (8 μm thickness) were dried for 1 h at room temperature. Fixation and staining were performed as described for whole organ processing. Counterstaining with Nuclear Fast Red (Sigma-Aldrich, St. Louis, MO) was performed before dehydration, clearing in Neo-clear (EM Science, Gibbstown, NJ), and coverslipping with Permount (Fisher Scientific, Santa Clara, CA).

For histological analysis, different organs from newborns were weighed and fixed by immersion in Bouin's fixative for 16 h. After dehydration and embedding, paraffin blocks were sectioned at 4 μm thickness and stained with hematoxylin and eosin using standard procedures.

LGR4 immunohistochemical analysis was performed using rabbit polyclonal antibodies against the ectodomain of the receptor. For LGR4 antibody production, cDNA corresponding to the ectodomain of human LGR4 was subcloned into the pcDNA3.1Zeo vector (Invitrogen, San Diego, CA) and stably transfected in 293T cells. The LGR4 ectodomain protein was purified using Nickel-affinity columns and emulsified in Freud's adjuvant before injection into rabbits to generate polyclonal antibodies. IgG was purified from bleeds using the Protein G Sepharose 4 Fast Flow column (Amersham Biosciences Corp., Piscataway, NJ). This specific LGR4 antiserum (dilution 1:1000) was used to localize LGR4 expression in wild-type newborn mice. Substitution of the primary antibody with the rabbit preimmune serum served as the negative control. Staining was performed using the Histostain-SPAEC kit following manufacturer's instructions (Zymed Laboratories, Inc., South San Francisco, CA).

Quantification of IGF-I, IGF-II, and IGFR-I Transcript Levels Based on Real-Time PCR Analysis

mRNAs were extracted from different organs using the RNAeasy kit (QIAGEN, Valencia, CA). cDNA was synthesized with the Omniscript reverse transcriptase (QIAGEN) using the oligo-dT primer (Invitrogen). PCR was performed using SmartCycler (Cepheid, Inc., Sunnyvale, CA) according to the manufacturer's protocol. The primers and probes for real-time PCR were as follows. IGF-I: sense, 5′-GATACACATCATGTCGTCTTCACAC-3′; antisense, 5′-GAACTGAAGAGCATCCACCAG-3′; probe, 5′-6-carboxy-fluorescein (FAM)-CTCTTCTACCTGGCGCTCT-GCTTG-6-carboxy-tetramethyl-rhodamine (TAMRA)-3′; IGF-II: sense, 5′-GACCGCGGCTTCTACTTCAGCA-3′; antisense, 5′-GGAAGCAGCACTCTTCCACGATG-3′; probe, 5′-FAM-CTTC-AAGCCGTGCCAACCGT-TAMRA-3′; IGFR-I: sense, 5′-GAC-ATCTACGAGAC-GGACTACTACC-3′, antisense, 5′-AGAATG-AGTAGTGAAGACACCATCC-3′; probe, 5′-FAM-TGCCTGTG-CGCTGGATGTCT-TAMRA-3′; and β-actin: sense, 5′-GGAC-CTGACGGACTACCTCATG-3′; antisense, 5′-TCTTTGATGTC-ACGCACGATTT-3′; probe, 5′-FAM-CCTGACCGAGCGTGGC-TACAGCTTC-TAMRA-3′. To determine the copy number of target transcripts, IGF-I, IGF-II, IGFR-I, and β-actin cDNAs were used to generate calibration curves by plotting the threshold cycle (Ct) *vs.* the known copy number for each plasmid template. The copy numbers for target samples were determined according to the calibration curve. To correct for differences in RNA extraction, data were normalized by dividing the copy number of the target cDNA by that of β-actin.

Blood and Urine Analysis

Blood was collected from the aorta of newborn mice in 50-μl nonheparinized capillary tubes. After clotting of blood, serum samples were analyzed for AST, glucose and BUN content after 1:5–1:20 dilutions. Urine was collected in 50-μl capillary tubes after gentle massage of the bladder. Urine samples were also analyzed for their BUN and creatinine content. All tests were performed by Stanford's Veterinary Service Center's diagnostic laboratory using standard procedures.

Data Analysis

All experimental data are presented as the mean ± SEM. Statistical significance was determined by ANOVA for multiple-group comparisons and by Tukey's post-test. Significance was accepted at $P < 0.05$.

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Neonatal Lethality of LGR5 Null Mice Is Associated with Ankyloglossia and Gastrointestinal Distension

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The physiological role of an orphan G protein-coupled receptor, LGR5, was investigated by targeted deletion of this seven-transmembrane protein containing a large N-terminal extracellular domain with leucine-rich repeats. LGR5 null mice exhibited 100% neonatal lethality characterized by gastrointestinal tract dilation with air and an absence of milk in the stomach. Gross and histological examination revealed fusion of the tongue to the floor of oral cavity in the mutant newborns and immunostaining of LGR5 expression in the epithelium of the tongue and in the mandible of the wild-type embryos. The observed ankyloglossia phenotype provides a model for understanding the genetic basis of this craniofacial defect in humans and an opportunity to elucidate the physiological role of the LGR5 signaling system during embryonic development.

The leucine-rich repeat-containing, G protein-coupled receptors (LGRs) designated LGR4 through LGR8 are structurally similar to receptors for gonadotropins and thyrotropin (11, 12). These receptors are characterized by a large N-terminal extracellular domain containing leucine-rich repeats followed by a seven-transmembrane region. Phylogenetic analyses showed three LGR subgroups: the glycoprotein hormone receptors; the subgroup of LGR4, LGR5, and LGR6; and a third subgroup represented by LGR7 and LGR8 recently found to be receptors for the relaxin family ligands (13, 15, 23). Evolutionary analyses suggested that these three subgroups of LGRs existed before the divergence of vertebrates and invertebrates (10). LGR5, also known as GPR49, HG38, or FEX, is a large protein consisting of 18 extracellular leucine-rich repeats together with a seven-transmembrane region (8, 12, 17). Unlike LGRs in the other two subgroups, the ligands and physiological functions for the LGR4, LGR5, and LGR6 genes are unclear.

Ankyloglossia is a rare human craniofacial defect associated with difficulties in an infant's ability to breast-feed and defective speech articulation (6, 16, 18). In patients with this disorder, the lingual frenulum, which attaches the tongue to the floor of the oral cavity, extends to the tip of the tongue, thereby preventing optimal tongue movement. Limitation of tongue movement may vary from very mild to complete fusion of the tongue to the floor of the mouth. Ankyloglossia in breast-feeding infants can cause ineffective latch, inadequate milk transfer, and maternal nipple pain, resulting in slower weight gain and untimely weaning. Some cases of ankyloglossia are associated with cleft palate (CPX; MIM 303400; OMIM database, Johns Hopkins University, Baltimore, Md.) and are inherited as an X-linked disorder caused by mutations in *TBX22*, a T-box transcription factor gene located in Xq21 (3).

In an attempt to elucidate the physiological roles of the subgroup of orphan LGRs consisting of LGR4, LGR5, and LGR6, we performed gene deletion experiments with LGR5 using a mouse model. Here, we report that the LGR5 null mice exhibit neonatal lethality associated with ankyloglossia characterized by fusion of the tongue to the floor of the mouth leading to the inability to nurse and neonatal mortality.

MATERIALS AND METHODS

Generation and genotyping of LGR5 null mice. A targeting vector was constructed in which exon 18 of the LGR5 gene was replaced with a neomycin resistance gene derived from the MC1 neo vector (Fig. 1A). Lex-1 embryonic stem (ES) cells were electroporated with the targeting vector before selection of the cells expressing the targeted allele for the generation of chimeric mice. LGR5 mutant mice were obtained by mating chimeric mice with C57BL/6 mice. Mice were housed in the Stanford Research Animal Facility, and all procedures were approved by the Institutional Care and Use Committee. Intercross litters were obtained by pairing heterozygous male and female animals overnight. The day the vaginal plug was observed was considered as embryonic day 0.5 (E0.5).

For genotyping, genomic DNA was extracted from tail tips of neonates with a genomic DNA extraction kit (Promega, Madison, Wis.). PCR was carried out with three primers (Fig. 1A): upstream primer A (5′-CCTCTTTGCTAAACCT CACC-3′) located in exon 18 of LGR5; the second upstream primer, B (5′-GC AGCGCATCGCCTTCTATC-3′), located at the 3′ end of the targeting vector; and the downstream primer, C (5′-ACGAGTCTTCTCACTATGGG-3′), located at the 3′ end of the targeting vector. Two PCR products were expected: a 673-kb product from wild-type alleles amplified by primers A and C and a 467-kb product from the targeted alleles amplified by primers B and C.

Estimation of LGR5 transcript levels based on real-time RT-PCR. Total RNA was extracted from different organs by employing the RNAeasy kit (QIAGEN, Valencia, Calif.) before cDNA synthesis using Advantage reverse transcriptase (BD Bioscience, Franklin Lakes, N.J.) and the oligo(dT) primer (BD Bioscience, Franklin Lakes, N.J.). PCR was performed in the SmartCycler (Cepheid, Inc., Sunnyvale, Calif.) according to the manufacturer's protocol. To test if the transgene containing the N-terminal extracellular domain (ectodomain) of LGR5 was expressed in the LGR5 null mice, we performed additional reverse transcription-PCR (RT-PCR) by amplifying the LGR5 ectodomain region with two pairs of primers: sense primers a (5′-CCTCTGCTTCCTAGAAGAGTTAC-3′) and c (5′TCAGTATGAACAACATCAGTCAG-3′) and antisense primers b (5′-CTA

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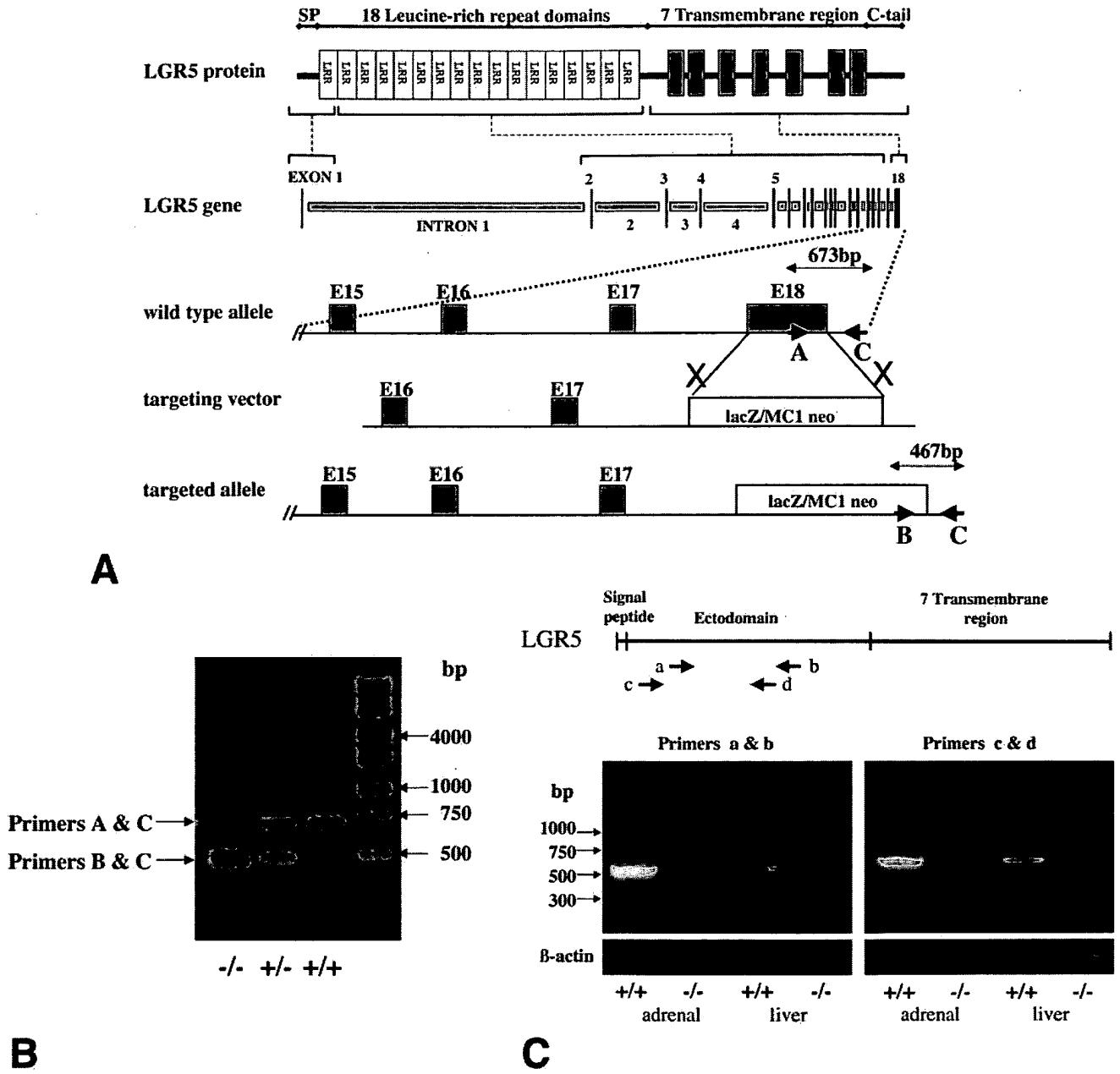

FIG. 1. Targeted disruption of the LGR5 genes and genotyping of LGR5 null mice. (A) Schematic representation of LGR5 genomic DNA, the targeting vector, and the disrupted gene. The LGR5 gene contains 18 exons, with exon 1 encoding the signal peptide for secretion (SP) and the N-terminal leucine-rich repeat domain. Exons 2 to 17 encode the 17 leucine-rich repeat (LRR) domains, and exon 18 encodes the seven-transmembrane region plus the C-terminal tail. In the targeting vector, the transgene containing the LacZ-MC1 neo cassette replaced exon 18 of the LGR5 gene, leading to the loss of LGR5 expression in the targeted allele. (B) PCR genotyping of wild-type (+/+), heterozygous (+/−), and LGR5 null (−/−) mice. Triplex PCR was performed with genomic DNA as the template together with three primers. Primers A and C allowed the amplification of an LGR5 gene fragment (673 bp) in the wild-type allele, whereas primers B and C amplified a chimeric gene fragment (467 bp) in the mutant allele. In heterozygous animals, both PCR products were generated. (C) Lack of expression of the LGR5 ectodomain-LacZ transgene in LGR5 null mice. (Top panel) Location of two sets of primers used to amplify the ectodomain of LGR5. (Lower panel) Amplification of LGR5 transcripts (a and b primers, 524 bp; c and d primers, 540 bp) in wild type (+/+) but not LGR5 null (−/−) mice. The levels of β-actin serve as positive controls.

GTTCCTTAAGGTTGGAGAGT-3') and d (5'-TTGCAGTGGGGAATTCAT CAAGGTTATTAT-3'). The primers for β-actin were sense primer 5'-GGACC TGACGGACTACCTCATG-3' and antisense primer 5'-TCTTTGATGTCACG CACGATTT-3'. The following primers and probes were used for real-time PCR: LGR5 sense 5'-CTTCCGAATCGTCGATCTTC-3' and antisense 5'-AACGAT CGCTCTCAGGCTAA-3', probe 5'-6-carboxy-fluorescein (FAM)-TCACTCT GGCAGCGCTGGAA-6-carboxy-fluorescein (FAM)-CTCTTCTACCTGGCG CTCTGCTTG-6-carboxy-tetramethyl-rhodamine (TAMRA)-3', β-actin sense 5'-GGACCTGACGGACTACCTCATG-3' and antisense 5'-TCTTTGATGTC ACGCACGATTT-3', and probe 5'FAM-CCTGACCGAGCGTGGCTACAGC TTC-TAMRA-3'. To determine the copy number of target transcripts, LGR5 and β-actin cDNAs were used to generate calibration curves by plotting the

TABLE 1. Neonatal lethality of LGR5 null mice[a]

Parameter	Mouse group			Total
	Wild type	Heterozygous	Homozygous	
No. of pups	32	74	35	141
No. of neonatal deaths	2	4	35	41
Body wt at birth (g)	1.45 ± 0.18	1.48 ± 0.17	1.39 ± 0.12	

[a] Genotyping was performed for intercrosses of heterozygous mice at the day of birth. In a total of 141 mice from 15 litters, the ratio of wild-type, heterozygous, and LGR5 null mice was 1:2.3:1.1. Although wild-type and heterozygous mice showed limited neonatal lethality (2 of 32 = 6.3% and 4 of 74 = 5.4%, respectively), all 35 of the LGR5 null newborns died within 24 h of birth.

threshold cycle (Ct) versus the known copy number for each plasmid template. The copy numbers for target samples were determined according to the calibration curve. To correct for differences in RNA extraction, data were normalized by dividing the copy number of the target cDNA by that of β-actin.

Expression of recombinant antigenic epitopes for LGR5 and generation of LGR5 antibody. The primary sequences encoding the ectodomain of human LGR5 cDNA were used to predict their antigenicity (14). A region corresponding to amino acids 22 to 141 of LGR5 was amplified from the plasmid containing full-length LGR5 cDNA and subcloned into the pET21a vector (Novagen, EMD Biosciences, San Diego, Calif.). Primer pairs for the selected epitope were designed as follows: sense 5′-GGCAGCTCTCCCAGGTCT-3′ and antisense 5′-GCAGAATTTGCGAAGCCTTCAA-3′. Expression of recombinant protein was induced in *Escherichia coli* strain BL21*trxB*(DE3) (Novagen, EMD Biosciences) with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 4 h. Recombinant protein with six-His tag was then purified from the inclusion body dissolved in 8 M urea by metal chelate chromatography. The protein was emulsified in Freund's adjuvant before injection into rabbits to generate polyclonal antibodies to LGR5.

Generation of the ectodomain of LGR5 in eukaryotic cells and immunoblotting analyses. A eukaryotic cell expression plasmid encoding the entire ectodomain (amino acid residues 22 to 508) of human LGR5 was transfected into human 293T cells. Clonal cell lines stably expressing a recombinant protein encoding the ectodomain of LGR5, named 5BP, were grown in Dulbecco's modified Eagle's medium (DMEM) combined with F-12 medium (DMEM/F-12) with 10% fetal bovine serum. After the cells were confluent, media were replenished with serum-free DMEM/F-12. Three days later, the media were collected, centrifuged, and filtered through 0.22-μm-pore-diameter filters (Corning, Cambridge, Mass.). Conditioned media containing recombinant 5BP were then purified by metal chelate chromatography. To verify the specificity of the LGR5 antibody, conditioned media containing the soluble 5BP were analyzed with immunoblots. Samples were fractionated by electrophoresis in a 7.5% polyacrylamide gel. Some samples were pretreated with *N*-glycosidase F (New England Biolabs, Inc., Beverly, Mass.) to confirm the glycoprotein nature of 5BP. Proteins were transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham Pharmacia Biotech, Piscataway, N.J.). Subsequently, immunoblotting was performed with rabbit polyclonal antibodies to LGR5 followed by incubation with a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G before immunofluorescent imaging with the ECL enhanced chemiluminescence Western blot system (Amersham).

Gross morphology as well as histological and immunohistochemical analyses. Newborn pups were euthanized with CO_2 and fixed in toto by immersion in Bouin's fixative for 16 h before paraffin embedding. Blocks were sectioned at a 4-μm thickness and stained with hematoxylin and eosin by standard procedures. Immunohistochemical analysis was performed with rabbit polyclonal antibodies against LGR5. Substitution for the primary antibody with rabbit preimmune serum served as the negative control. Staining was performed with the Histostain-SPAEC kit following the manufacturer's instructions (Zymed Laboratories, South San Francisco, Calif.).

Data analysis. All experimental data are presented as the mean ± standard error. Statistical analysis was performed with Statview 4.5 (Abacus Concepts, Berkeley, Calif.) software. Data were analyzed with the Mann-Whitney U test. Significance was accepted at $P < 0.05$.

RESULTS

Based on recombinant DNA targeting, the LGR5 gene was disrupted in mice following the replacement of exon 18 encoding the seven-transmembrane region with a transgene containing selection markers (Fig. 1A). ES cells expressing the targeted allele were selected to derive chimeric mice for

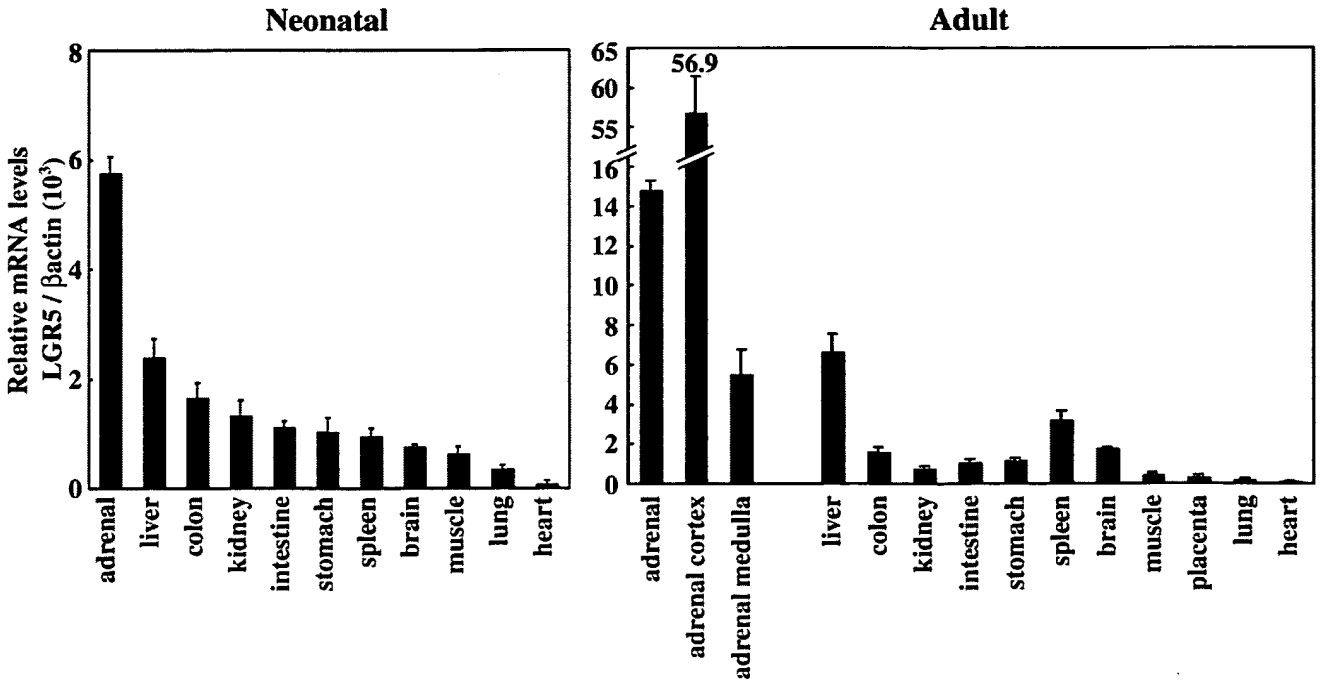

FIG. 2. Tissue expression pattern of LGR5 in mice. Real-time RT-PCR analyses were performed to estimate LGR5 transcript levels in diverse tissues from neonatal (left panel) and adult (right panel) mice. Results are expressed as the ratios between LGR5 and β-actin transcripts for normalization.

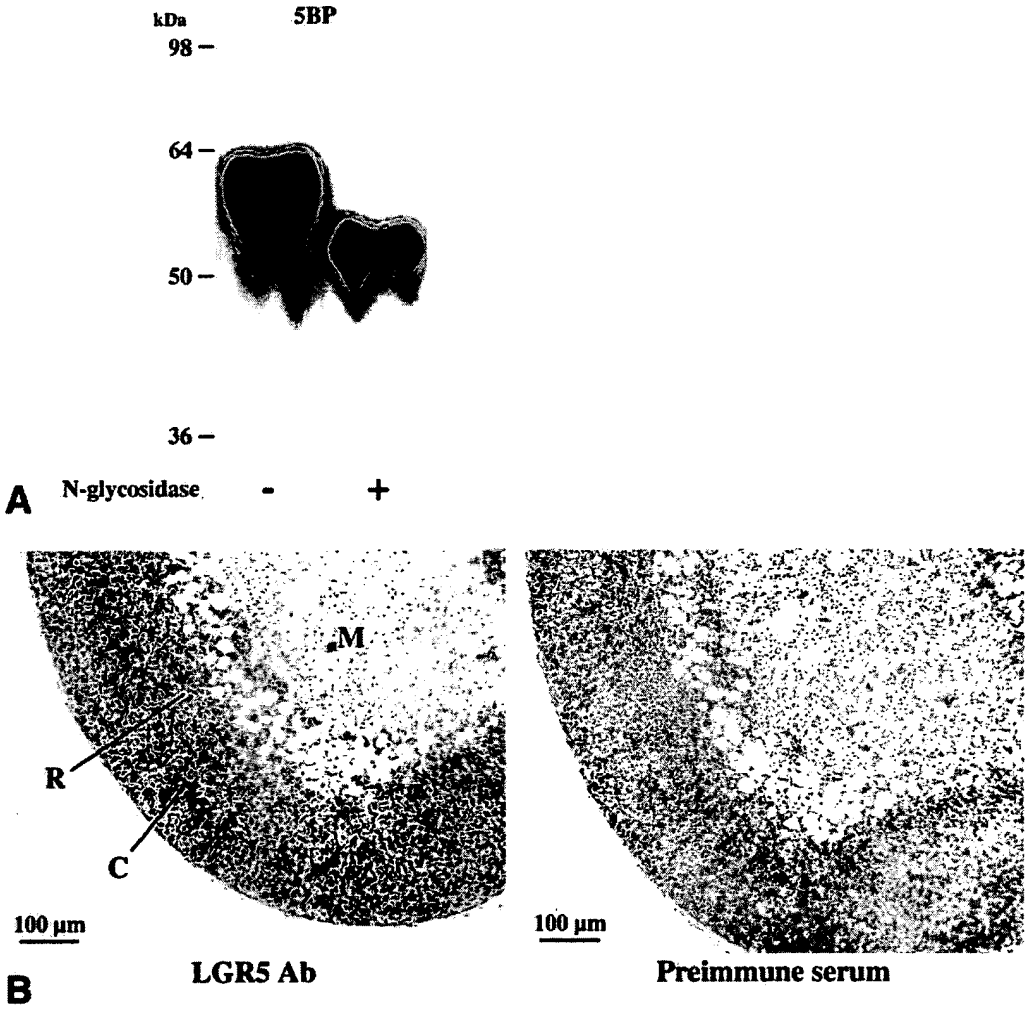

FIG. 3. Characterization of LGR5 antibodies and immunostaining of LGR5 in adrenal gland. (A) Immunoblot analyses of affinity-purified 5BP corresponding to the ectodomain of LGR5. Some samples were pretreated with *N*-glycosidase to remove N-linked carbohydrate side chains. (B) Immunostaining of LGR5 in the adrenal glands of adult mice. Ab, antibody; C, cortex; M, medulla; R, zona reticularis.

subsequent generation of LGR5 null mice. Genotyping was performed at birth by multiplex PCR analyses to amplify LGR5 gene fragments with or without the transgene (Fig. 1B). Because our β-galactosidase staining in mutant mice suggested a lack of expression of the transgene encoding the ectodomain of LGR5 fused to LacZ (data not shown), we further performed RT-PCR analyses in neonates to completely rule out the expression of LGR5 transcripts in mutant animals. Using two pairs of primers corresponding to the ectodomain of LGR5, RT-PCR analyses indicated a lack of expression of this region in LGR5 null mice as compared with the high expression in adrenal gland and liver of wild-type littermates (Fig. 1C). Comparable levels of expression of the β-actin transcripts in both mutant and wild-type animals served as positive controls.

Based on the expected Mendelian ratio of wild-type and mutant animals following mating of heterozygous mice, embryonic lethality of LGR5 null mice could be excluded (Table 1). Although no significant difference in body weight was observed at birth among different groups of animals ($P > 0.05$), all the LGR5 null mice died within 24 h after parturition.

To further understand the role that deletion of LGR5 plays in the death of LGR5 null mice, the expression of LGR5 in diverse tissues was analyzed by performing real-time PCR and immunostaining in wild-type mice. As shown in Fig. 2, LGR5 transcripts were detected in multiple tissues, with adrenal glands and liver showing the highest levels in both neonatal and adult animals. In adrenal glands, the LGR5 transcript was higher in the cortex but significantly lower in the medulla. We further generated a fragment (amino acids 22 to 141) of LGR5 in prokaryotic cells and purified the epitope-tagged protein for the generation of LGR5 antibodies. As shown in Fig. 3A, immunoblotting utilizing this antibody detected the purified recombinant 5BP derived from eukaryotic cells as an N-linked glycoprotein. Using this antibody, immunohistochemical analyses indicated that LGR5 was highly expressed in the outer adrenal cortex, with minimal levels in the medulla and zona reticularis (Fig. 3B). Although the LGR5 gene was expressed in adrenal gland, no difference in the morphology of the gland was found in LGR5 null newborn mice (data not shown). Likewise, no abnormal liver histology was apparent.

Close examination of the LGR5 null neonates revealed a

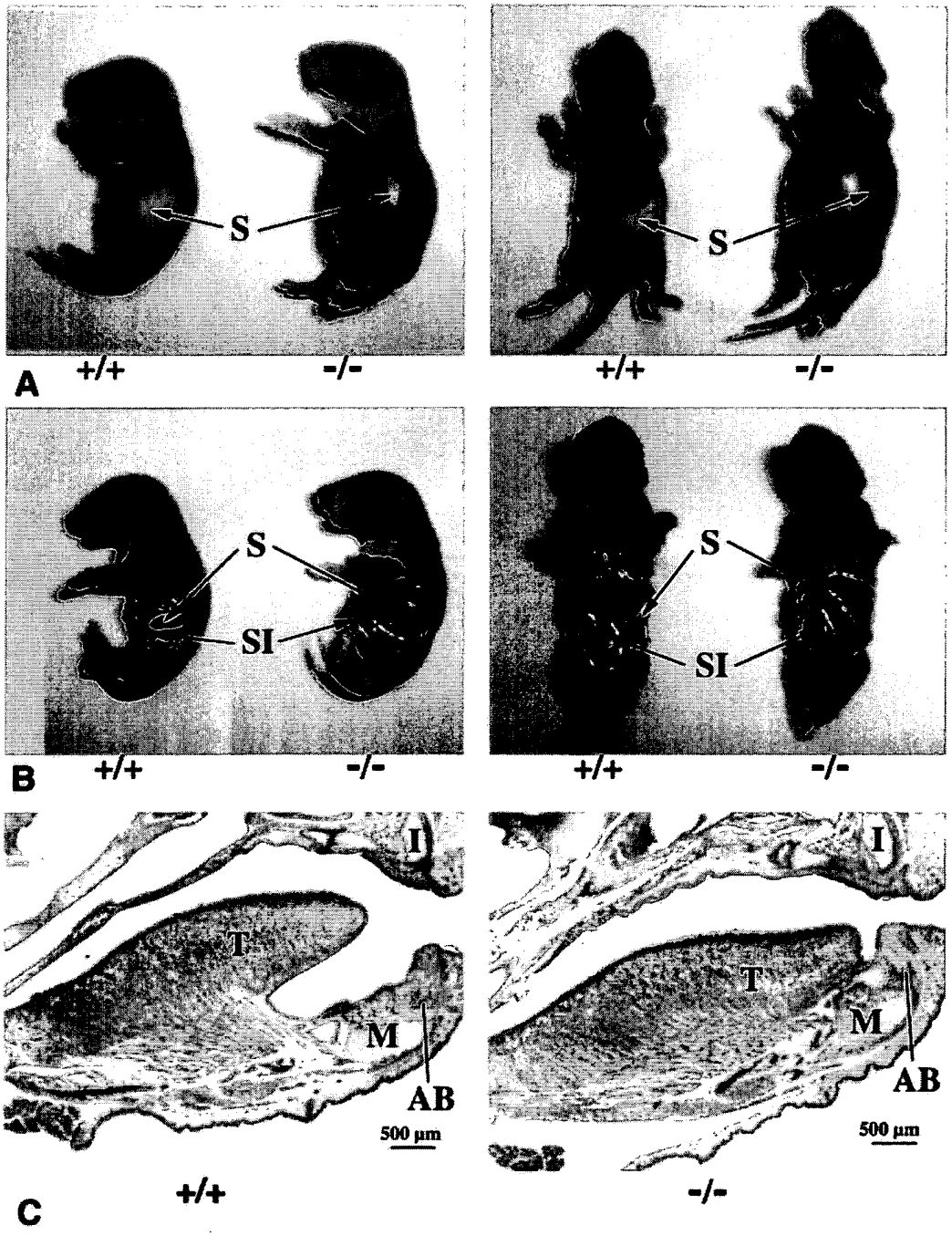

FIG. 4. Dilated gastrointestinal tract and abnormal craniofacial development in LGR5 null mice. (A) The wild-type LGR5 (+/+) neonates had milk in their stomachs (S), whereas the LGR5 null (−/−) neonates had dilated stomachs without milk. (B) The entire gastrointestinal tract of LGR5 null mice was dilated without milk, whereas the wild-type mice had a normal appearance. SI, small intestine. (C) In sagittal sections of the craniofacial region, the LGR5 null mice showed fusion of the tongue (T) to the mandible (M), whereas these two regions are separated in the wild-type mice. Identification of similar structures in the upper and lower jaws indicates the sections were taken at the same level. I, developing upper incisor; AB, developing alveolar bone of lower incisor. (D) In transverse serial sections of the mandible region, the tongue of LGR5 null mice was attached to the mandible, whereas the tongue of the wild-type animals in the same region was connected only in a posterior section. Similar sections are reflected by the morphology of the molar teeth (mt), and more anterior sections are shown on the right. (E) Immunostaining of LGR5 antigen in the epithelium of the tongue and the epithelium and mesenchyme of the mandible at E14.5. Ab, antibody. The boxed area in the upper panel is enlarged in the lower panels.

gradual distension of the abdomen after parturition. As shown in Fig. 4A, wild-type animals had milk in their stomachs but the stomachs of LGR5 null mice were empty and filled with air, presumably as the result of aerophagia associated with a suckling defect. The mutant animals became gradually cyanotic with gasping respirations. By 12 h after birth, the entire gastrointestinal tract had become distended (Fig. 4B). The perinatal lethality of these animals was due partly to respiratory

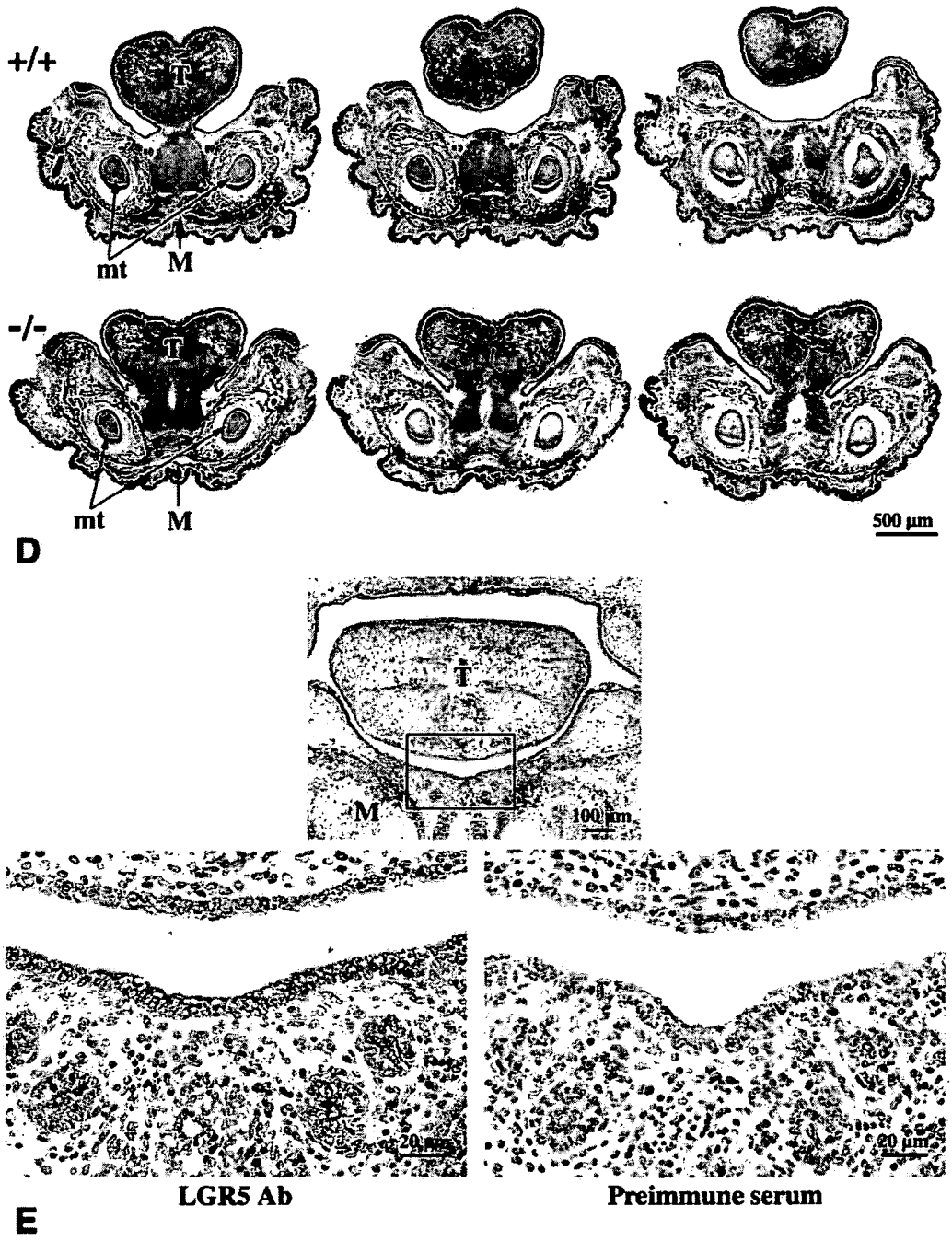

FIG. 4—*Continued.*

failure as the result of pressure against the diaphragm from the markedly distended abdomen. Because earlier studies in mice linked craniofacial defects such as cleft palate to suckling-related neonatal lethality (1, 6, 7, 21), we searched for possible anatomical abnormalities in LGR5 mutant mice. Although a cleft palate was not found, gross examination of the oral cavity revealed immobilized tongues adherent to the floor of the oral cavity. Histological sagittal sections of the head revealed that the tongue in LGR5 null mice was fused along the entire ventral surface to the floor of the oral cavity, in contrast to the expected conformation as seen in wild-type mice (Fig. 4C). In transverse serial sections of the mandible region, persistent attachment of the tongue proper of the mandible was also apparent (Fig. 4D). To determine if LGR5 proteins were expressed in this region during early development, immunostaining was performed in transverse sections from wild-type embryos at E14.5. As shown in Fig. 4E, LGR5 staining could be found in the epithelium of the tongue and the epithelium and mesenchyme of the mandible.

DISCUSSION

The present studies in LGR5 null mice indicated that this orphan receptor is involved in craniofacial development and the mutant mice provide an animal model for the investigation of the genetic basis of ankyloglossia. All LGR5 null mice died within 24 h of birth. This phenotype is similar to that of neonatal mice with a cleft palate. Although for humans, cleft palate is not life-threatening, mice with cleft palate die within 24 h of birth, showing an abdomen dilated with air due to a suckling defect (1, 7, 21). In humans, ankyloglossia is reported to cause breast-feeding difficulties and results in slower weight gain (2, 24). Although there are no reports describing mice with ankyloglossia unaccompanied by cleft palate, the observed neonatal lethality of all LGR5 null mice suggests that ankyloglossia alone could prevent proper suckling in mice.

During embryonic development, the tongue is formed from foregut endoderm and by E13 the distal end of the tongue is freed from the floor of the mouth. Programmed cell death and resorption of the developing skeletal muscle in the ventral anterior region free the tongue, and normally a thin tissue band, the lingual frenulum, remains as the only attachment. Disturbances of this process result in an anteriorily extended and/or shortened frenulum leading to the ankyloglossia phenotype. Findings of ankyloglossia in LGR5 mutant mice together with the expression of LGR5 in the epithelium of the tongue and tissues within the mandible of wild-type animals suggest a role of the LGR5 signaling pathway in proper tongue development. Our immunohistochemical localization of LGR5 in the tongue and the mandible in wild-type mice is consistent with an earlier report based on in situ hybridization analyses (8). At E11.5, LGR5 transcripts were found in the epithelium and mesenchyme overlaying the mandibular cleft. At E13.5, the signal persisted in this region together with signals in the most lateral aspects of the tongue.

Although no cleft palate was found in LGR5 null mice, mutations in a transcriptional factor, TBX22, have been reported in families with both cleft palate and ankyloglossia (3). Of interest, in situ hybridization analyses of *Tbx22* expression in mouse embryos indicated that this gene is expressed in the mesenchyme of the inferior nasal septum, the posterior palatal shelf before fusion, the base of the tongue, and lateral region of the mandible (4, 9). Although *Tbx22* mutant mice have not been reported, the localization of *Tbx22* in the lingual frenulum correlated with the ankyloglossia phenotype in humans. Many different ligand signaling systems have been implicated in craniofacial development. These include several transforming growth factor β (TGF-β) ligands, fibroblast growth factors (FGFs), hedgehog paralogs, wingless genes, platelet-derived growth factors, and endothelin-1 (6). Recent studies further emphasized the involvement of FGF, sonic hedgehog, and TGF-β/BMP ligand signaling systems in palate formation (19). Of interest, both FGF receptor 2b (FGFR2b) and FGF10 null mice exhibited cleft palate and a partial ankylosis of the tongue associated with defective epithelialization between the floor of the mouth and the tongue (22). The present observations of the ankyloglossia phenotype in LGR5 null mice underlie important roles of G protein-coupled receptors during tongue formation and suggest possible interactions of diverse ligand signaling systems during craniofacial development. The present study provides the first mouse model to understand the human ankyloglossia phenotype without associated cleft palate.

In addition to expression in the craniofacial region, LGR5 transcripts have been found in other tissues. Northern blot analysis showed that LGR5 is expressed in skeletal muscle, placenta, spinal cord, and various regions of the brain (12, 17). In situ hybridization analyses further demonstrated the expression of LGR5 in adrenal medulla, male and female gonads, and the olfactory bulb of adult mice (8). The present LGR5 expression analyses, in general, confirmed these earlier reports. However, we detected both LGR5 transcripts and antigens mainly in adrenal cortex, inconsistent with an earlier in situ hybridization analysis showing high LGR5 transcripts in adrenal medulla (8). The basis for these discrepancies is unclear.

In addition to its role in craniofacial formation during embryonic development, LGR5 may also play important roles in adult life. A recent study demonstrated the overexpression of LGR5 in human hepatocellular carcinomas with β-catenin mutations, suggesting that LGR5 may be involved in tumorigenesis (25). Future identification of the cognate ligand for LGR5 and the elucidation of the signaling pathway for this G protein-coupled receptor could provide a better understanding of the molecular mechanisms of tongue development and the physiological roles of this subgroup of LGRs conserved between vertebrates and invertebrates (5, 20).

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Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery

Jeffrey M. Stadel, Shelagh Wilson and Derk J. Bergsma

Access to DNA databases has introduced an exciting new dimension to the way biomedical research is conducted. 'Genomic research' offers tremendous opportunity for accelerating the identification of the cause of disease at the molecular level and thereby foster the discovery of more selective medicines to improve human health and longevity. The current challenge is to close the gap rapidly between gene identification and clinical development of efficacious therapeutics. In the present review, **Jeffrey Stadel, Shelagh Wilson and Derk Bergsma** outline the rationale and describe strategies for converting one large class of novel genes, orphan G protein-coupled receptors (GPCRs), into therapeutic targets. Historically, the superfamily of GPCRs has proven to be among the most successful drug targets and consequently these newly isolated orphan receptors have great potential for pioneer drug discovery.

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The advent of rapid DNA sequencing spawned the 'genomic era', which has led to the initiation of the Human Genome Project. The novel technologies developed in association with genomic research have already had a significant impact on the way investigations into the basis of disease are being conducted and will, no doubt, substantially enhance the means by which diseases are diagnosed and treated in the near future. To keep pace with the evolution of molecular medicine, the pharmaceutical industry has embraced genomics and is attempting to exploit the new technologies to identify novel targets for drug discovery. The major questions that remain to be addressed concern how to convert genomic sequences into therapeutic targets in an expeditious manner and eventually to obtain pharmaceutical drugs that will enhance the quality of life. This review will deal with a single class of novel molecular targets, focusing on the burgeoning collection of G protein-coupled receptors (GPCRs) called 'orphan' receptors[1]. GPCRs are a superfamily of integral plasma membrane proteins involved in a broad array of signalling pathways. Since the first cloning of GPCR gene sequences over a decade ago, novel members of the GPCR superfamily have continued to emerge through cloning activities as well as through bioinformatic analyses of sequence databases, although their ligands are unidentified and their physiological relevance remain to be defined. These 'orphan' receptors provide a rich source of potential targets for drug discovery.

The members of the GPCR superfamily are related both structurally and functionally. The signature motif of these receptors is seven distinct hydrophobic domains, each of which is 20–30 amino acids long and which are linked by hydrophilic amino acid sequences of varied length[2,3]. Biophysical[4] and biochemical[5] studies support the notion that these receptors are intercalated into the plasma membrane with the amino terminus extracellular and the carboxy terminus in the cytoplasmic portion of the cell. Therefore, these receptors are often referred to as seven transmembrane (or 7TM) receptors. While it is not yet known how many individual genes actually encode these receptors, it is clear that this family of proteins is one of the largest yet identified. Functionally, GPCRs share in common the property that upon agonist binding they transmit signals across the plasma membrane through an interaction with heterotrimeric G proteins[6,7]. These receptors respond to a vast range of agents[2,5,8] such as protein hormones, chemokines, peptides, small biogenic amines, lipid-derived messengers, divalent cations (e.g. a Ca^{2+} sensor has been identified that is a GPCR)[9] and even proteases such as thrombin, which activates its receptor by cleaving off a portion of the amino terminus[10]. Finally, these receptors play an important role in sensory perception including vision and smell[2,5,8]. Correlated with the broad range of agents that activate these receptors is their existence in a wide variety of cells and tissue types, indicating that they play roles in a diverse range of physiological processes. It is likely, therefore, that the GPCR superfamily is involved in a variety of pathologies. This point was recently emphasized by the surprising discovery that certain GPCRs for chemokines act as co-factors for HIV infection[11–13].

GPCRs represent the primary mechanism by which cells sense alterations in their external environment and convey that information to the cells' interior. The binding of an agonist to the receptor promotes conformational changes in the cytoplasmic domains that lead to the interaction of the receptor with its cognate G protein(s). Agonist-promoted coupling between receptors and G proteins leads to the activation of intracellular effectors that substantially amplify the production of second messengers feeding into the signalling cascade. Since effectors are often enzymes [e.g. adenylate cyclase[14], which converts ATP to cAMP, or phospholipase C (Ref. 15), which hydrolyses inositol lipids in membranes to release inositol trisphosphate, which in turn mobilizes Ca^{2+} within a cell] or ion channels[16], many second messenger molecules can be produced as the result of a single agonist binding event with its receptor. Changes in the intracellular levels of ions or cAMP, or both,

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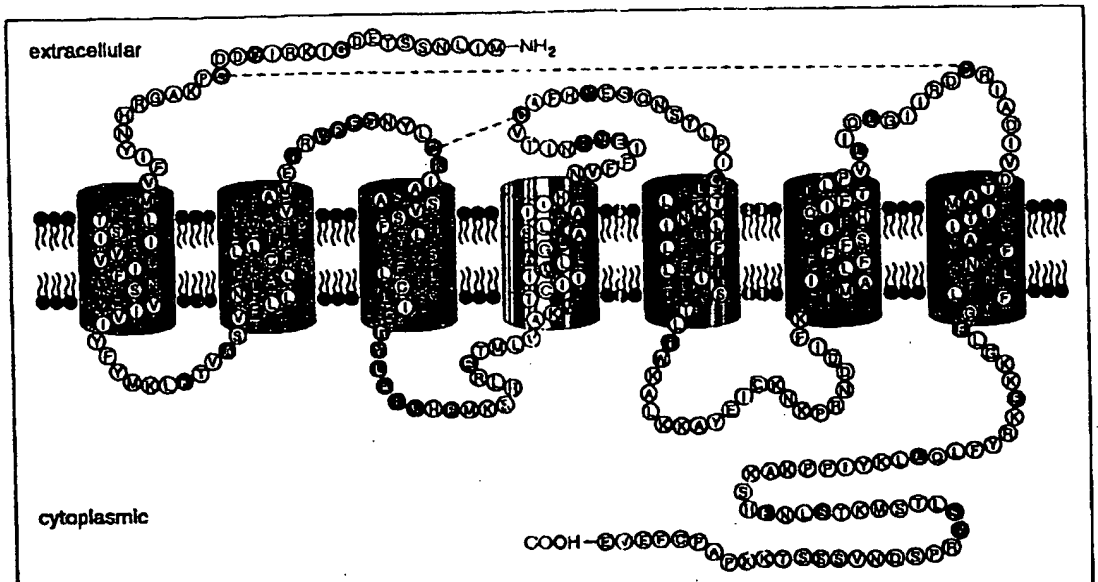

Fig. 1. Comparison of the protein sequence identity of the orphan APJ[15] receptor with the angiotensin AT_1 receptor[20]. The filled circles indicate amino acid identity (29.9%) between the two G protein-coupled receptors (GPCRs). This is a typical example of the protein sequence identity shared between orphan and known GPCRs.

result in the modulation of distinct phosphorylation cascades[17,18], extending through the cytosol to the nucleus, that eventually culminate in the physiological response of the cell to the extracellular stimulus. Although the overall paradigm is apparently the same for all GPCRs, the diversity of receptors, G proteins and effectors suggest a myriad of potential signalling processes and this becomes an important concept as we try to identify the function of orphan GPCRs.

To date, more than 800 GPCRs have actually been cloned from a variety of eukaryotic species, from fungi to humans [see L. F. Kolakowski in GCRDb-WWW The G Protein-Coupled Receptor DataBase World-Wide-Web Site (http://receptor.mgh.harvard.edu/GCRDBHOME.html.org)]. For humans, the most represented species, about 140 GPCRs have been cloned for which the cognate ligands are also known. This number excludes the sensory olfactory receptors, of which hundreds to thousands are predicted to exist. By traditional molecular genetic approaches, coupled with the explosion in genomic information, it has been possible to identify more than 100 additional orphan GPCR family members. By definition, there is enough sequence information in the receptor cDNAs to place them clearly in the superfamily of GPCRs, but often there is insufficient sequence homology with known members of this family to be able to assign their ligands with confidence or predict their function. In total, there are currently over 240 human GPCRs, excluding sensory receptors. As the size of sequence databases continues to increase, this list is expected to grow to 400, and perhaps even to 1000 or more unique gene products. The list will grow even further as paralogues and alternatively spliced GPCR variants emerge. Most orphan GPCRs share a low degree of sequence homology (typically about 25–35% overall amino acid sequence identity), with known GPCRs, suggesting that they belong to new subgroups of receptors (Fig. 1)[19,20]. Indeed, several orphan GPCRs show closer homology to each other than to known GPCRs. Nevertheless, the majority of orphan receptors are phylogenetically distributed among a broad spectrum of distantly related, known receptor subgroups.

What is the rationale for investing considerable time and resources into trying to establish the function of orphan GPCRs? Simply stated, GPCRs have a proven history of being excellent therapeutic targets. Within the past 20 years, several hundred new drugs have been registered that are directed towards activating or antagonizing GPCRs; in fact, it is estimated that most current research within the pharmaceutical industry is focused on this signalling pathway[21]. Table 1 shows a representative snapshot of a variety of receptors, disease targets and corresponding drugs. It is clear from this table that the therapeutic targets span a wide range of disorders and disease states. Another example of the significance and versatility of GPCRs is the number of cases of genetic diseases that are linked to defects in these proteins; some of these diseases are indicated in Table 2 (Refs 22–38). It is likely that many more genetic diseases will be mapped to GPCRs as the era of genomics continues to expand and families with inherited mutations are examined much more comprehensively.

The importance of GPCRs to drug discovery continues to be manifested by the fact that across the pharmaceutical industry active research projects, ranging from basic studies all the way through to advanced development, are focused on GPCRs as primary targets. Molecular biology has had a dramatic influence on these efforts.

Table 1. Examples of marketed drugs for G protein-coupled receptors (GPCRs)

GPCR	Generic	Drug	Indication
Muscarinic acetylcholine	Bethanechol	Urecholine	GI
	Dicyclomine	Bentyl	GI
	Ipratropium	Atrovent	CP
Adrenoceptor			
β_1	Atenolol	Tenormin	CP
α_2	Clonidine	Catapres	CP
β_1/β_2	Propranolol	Inderal	CP
α_1	Terazosin	Hytrin	CP
β_2	Albuterol	Ventolin	CP
$\beta_1/\beta_2/\alpha_1$	Carvedilol	Coreg	CP
Angiotensin			
AT_1	Losartan	Cozaar	CP
	Eprosartan	Teveten	CP
Calcitonin	Calcitonin	Calcimar	Osteoporosis
	eel-Calcitonin	Elcatonin	Osteoporosis
Dopamine			
D_2	Metoclopramide	Reglan	GI
D_2/D_3	Ropinirole	Requip	CNS
D_2	Haloperidol	Haldol	CNS
Gonadotropin-releasing factor	Goserelin	Zoladex	Cancer
	Nafarelin	Synarel	Endometriosis
Histamine			
H_1	Dimenhydrinate	Dramamine	CNS
H_1	Terfenadine	Seldane	CP
H_2	Cimetidine	Tagamet	GI
H_2	Ranitidine	Zantac	GI
Serotonin (5-HT)			
$5\text{-}HT_{1D}$	Sumatriptan	Imitrex	CNS
$5\text{-}HT_{2A}$	Ritanserin	Tisertan	CNS
$5\text{-}HT_4$	Cisapride	Propulsid	GI
$5\text{-}HT_{1B}$	Trazodone	Desyrel	CNS
$5\text{-}HT_{2A/2C}$	Clozapine	Clozaril	CNS
Leukotriene	Pranlukast	Onon	CP
	Zafirlukast	Accolate	CP
Opioid			
κ	Buprenorphine	Buprenex	CNS
	Butorphanol	Stadol	CNS
μ	Alfentanil	Alfenta	CNS
	Morphine	Kadian	CNS
Oxytocin		Syntocinon	Labour
Prostaglandin	Epoprostenol	Flolan	CP
	Misoprostol	Cytotec	GI
Somatostatin	Octreotide	Sandostatin	Cancer
Vasopressin	Desmopressin		CP/Renal

CP, cardiopulmonary system; GI, gastrointestinal system.

Table 2. Diseases associated with mutations of G protein-coupled receptors (GPCRs)

GPCR	Mutation	Disease	Refs
Rhodopsin	Missense: Pro23 to His (NT) Missense: Val87 to Asp (2TM) Missense: Tyr178 to Cys (2EL) Nonsense: Gln344 to Stop (CT)	Retinitis pigmentosa	22, 23
Thyroid stimulating hormone	Missense: Asp619 to Gly (3IL) Missense: Ala623 to Ile (3IL)	Hyperfunctioning thyroid adenomas	24
Luteinizing hormone	Missense: Asp578 to Gly (6TM)	Precocious puberty	25
Vasopressin V_2	Missense: Arg137 to His (2IL) Missense: Gly185 to Cys (2EL) Frameshift at Arg230 (5TM)	X-linked nephrogenic diabetes	26–28
Ca^{2+}	Missense: Arg186 to Glu (NT) Missense: Glu298 to Lys (NT) Missense: Arg796 to Trp (3IL) Missense: Glu128 to Ala (NT)	Hyperparathyroidism, hypocalciuric hypercalcaemia	29, 30
Parathyroid hormone (PTH type b)	Missense: His223 to Arg (1IL)	Short-limbed dwarfism	31
β_3-Adrenoceptor	Missense: Trp64 to Arg (1IL)	Obesity, NIDDM	32–34
Growth-hormone-releasing hormone	Nonsense: Glu72 to Stop (NT)	Dwarfism	35
Adrenocorticotropin	Missense: Ser74 to Ile (2TM)	Glucocorticoid deficiency	36
Glucagon	Missense: Gly40 to Ser (NT)	Diabetes, hypertension	37, 38

Abbreviations: CT, carboxyl terminus; EL, extracellular loop; IL, intracellular loop; NIDDM, non-insulin-dependent diabetes mellitus; NT, amino terminus; TM, transmembrane segment.

The cloning of cDNAs for well-known GPCRs led to the discovery of a surprising number of paralogues[5]. The existence of these novel receptor subtypes was unexpected because the current cornucopia of pharmacological agents does not possess the required selectivity to distinguish all of them clearly, and thus an opportunity for drug discovery was quickly recognized. Current research efforts seek to define the physiology associated with these novel receptor subtypes and to discover highly selective compounds as potential pharmaceutical drugs. These efforts are almost exclusively focused on GPCRs for which activating ligands are known. Since characterized GPCRs were, and continue to be, attractive therapeutic targets, it is most reasonable to speculate that many of the orphan receptors have similar potential. The initial challenge is to determine the function of each orphan receptor through the identification of activating ligands and, once the function is clarified, link the orphan receptor to a specific disease and thus establish it as a candidate for a comprehensive drug discovery effort.

Reverse molecular pharmacology

Until recently, research into the identification of GPCRs as targets for drug discovery has been conducted using the traditional approach illustrated in Fig. 2. For this strategy, the starting point is functional activity, which forms the basis of an assay by which a ligand is identified through purification from biological fluids, cell supernatants or tissue extracts. One example of the success of this strategy is the discovery of the potent vasoconstricting peptide endothelin[39]. Once isolated, the ligand is used to characterize its cellular and tissue biology as well as its pathophysiological role. Subsequently, cDNAs encoding corresponding receptors are 'fished' from gene libraries using a variety of methodologies (e.g. receptor purification and expression cloning) that often either directly or indirectly use the ligand as the 'hook'. As the nucleotide sequences for GPCRs begin to accumulate and be analysed, additional receptors can be cloned by homology screening, by positional cloning, and by polymerase chain reaction (PCR) methodologies that use oligonucleotide primers based on nucleotide sequences conserved within the seven transmembrane domains of the GPCR family. Once the cloned human receptor cDNA is expressed in a heterologous cell system[40], it is used, together with its ligand, to form the basis of a screen to explore chemical compound libraries for receptor antagonists or agonists. Lead structures identified in the screen are refined through medicinal chemistry using an iterative process. Resulting drug leads with appropriate *in vivo* pharmacology are passed on into the clinic for development.

Recently, this paradigm has changed radically with the introduction of a new reverse molecular pharmacological

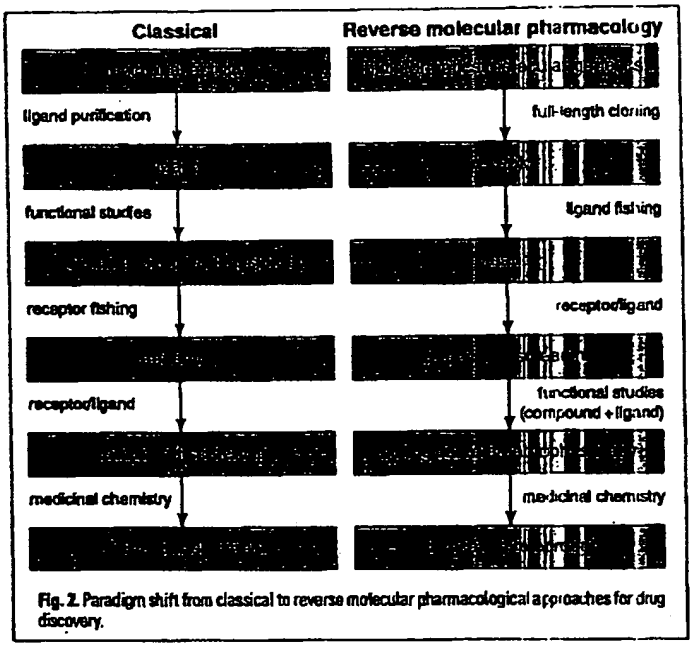

Fig. 2. Paradigm shift from classical to reverse molecular pharmacological approaches for drug discovery.

strategy, shown diagramatically in Fig. 2. Through both traditional molecular cloning techniques and, more recently, mass sequencing of expressed sequence tags (ESTs) from cDNA libraries, it is now possible to identify GPCRs through computational or bioinformatic methodologies. The EST approach, initially proposed by Sidney Brenner (University of Cambridge) and first brought to large-scale practice by Craig Venter (The Institute of Genome Research), constitutes random, single-pass sequencing of cDNAs randomly picked from a collection of cDNA libraries, followed by extensive bioinformatic analysis of the sequence to identify structural signatures characteristic of GPCRs. Once new members of the GPCR superfamily are identified, the recombinantly expressed receptors are used in functional assays to search for the associated novel ligands. The receptor–ligand pair are then used for compound bank screening to identify a lead compound that, together with the activating ligand, is used for biological and pathophysiological studies to determine the function and potential therapeutic value of a receptor antagonist (or agonist) in ameliorating a disease process. In addition, clues as to therapeutic potential may involve receptor genotyping of disease populations. Once a link with a disease is finally identified, an appropriate compound can be advanced for clinical study.

The reverse molecular pharmacological strategy is a far more daunting challenge and risky endeavour when compared with the more traditional approach, since the starting material for a drug discovery effort is simply an orphan receptor of unknown function, with no apparent relationship to a disease indication. However, the potential reward of using this approach is that resultant drugs naturally will be pioneer or innovative discoveries, and a significant proportion of these unique drugs may be useful to treat diseases for which existing therapies are lacking or insufficient.

Screening strategy

Figure 3 illustrates the generic strategy that we use for our reverse molecular pharmacological approach. In addition to the EST approach, which has yielded the majority of our collection of orphan receptors, we have also used a number of more traditional approaches such as low-stringency screening, using portions of known GPCRs as hybridization probes, as well as PCR-based methods. By these techniques we have succeeded in identifying more than 70 orphan receptors in addition to those already cited in the literature.

Since cDNAs identified by EST cloning are often incomplete, northern hybridization analysis is used to establish the tissue or cell pattern of mRNA expression of the GPCRs. This information is used to identify the tissue or cell cDNA libraries that are to be probed for full- length clones and, significantly, to determine whether a receptor is expressed in a particular normal or diseased tissue of interest. A highly selective tissue expression pattern may also provide a clue with respect to receptor function. Once obtained, full-length GPCR clones are expressed in mammalian cell lines and yeast model systems (see below) for functional analysis. *Xenopus* oocytes may also be used for expression; however, low screening throughput limits their use to a secondary, confirmatory assay system. For mammalian cell expression, the human embryonic kidney (HEK) 293 cell line or Chinese hamster ovary (CHO) cells are frequently used. These cell types possess a large repertoire of G proteins that are necessary for coupling to downstream effectors *in situ*. They also share a reliable history of positive functional coupling for a wide variety of known GPCRs. However, since receptor coupling cannot be accurately predicted from primary sequence data, orphan GPCRs may need to be expressed in a variety of cell lines to establish viable coupling.

These heterologous expression systems form the basis for screening for an activating ligand. The success of establishing functional coupling of the recombinant receptor depends to a large extent on whether the receptor is properly expressed, which may be assessed by northern or Western blot analysis, and whether appropriate G proteins and downstream effectors are present in the cell in which the receptor is expressed. There are several major technical challenges to be met in order to initiate ligand fishing. Because it is difficult to predict accurately the coupling specificity of orphan GPCRs from their primary sequence, assays must be chosen that will detect a wide range of coupling mechanisms. These generally focus on changes in intracellular levels of cAMP or Ca^{2+} but can also include more generic measurements, such as metabolic activation of the cell via the cytosensor microphysiometer[41]. Recently, it has become possible to implement most of these screens in high-throughput format by using fluorescent-based

assays and using charge-coupled device cameras and reporter gene constructs that allow easy readout of the assay on microtitre plates. Ever increasing throughput of the assays will be necessary to screen large libraries. However, this approach is somewhat cumbersome and inefficient if all the assays described above have to be used. Is it possible to funnel heterologous signal transduction through a defined pathway? The prospect of an assay for a single transduction pathway comes from the observation that heterologous expression of the G protein subunit $G_{\alpha 15/16}$ promoted coupling of various GPCR subfamily members through activation of phospholipase Cβ and likely Ca^{2+} mobilization[42,43]. Although this approach may not work universally, the diversity of the GPCRs successfully coupled through $G_{\alpha 16}$ to phospholipid metabolism suggests that this could be a useful method to screen for orphan receptor activation.

Once heterologous receptor expression is achieved and functional assays are in place, ligand fishing experiments can be initiated. Although the homology with known GPCRs is low, we nevertheless begin by screening the orphans against known GPCR ligands; since the sequence homology between some subtypes of known receptors can be low (e.g. 30–40% between neuropeptide Y receptor subtypes), it is possible that new paralogue receptors for known ligands still remain to be discovered. The next step is to search for novel activating ligands by screening biological extracts obtained from tissues, biological fluids and cell supernatants. An additional option is screening libraries of compounds for activating ligands. Complex libraries of peptides or compound collections could be rich sources of 'surrogate' agonists that would promote receptor activation and coupling but are not endogenous ligands. The rationale for searching for surrogate agonists springs from a report that a nonpeptide agonist has been discovered for the angiotensin II receptor[44]. There is also an obvious precedent for nonpeptide agonists for opioid receptors. Screening of the very large libraries that will be generated by fractionation of biological extracts and by combinatorial chemical synthesis requires that the functional assays used have not only a high throughput but are also robust, since false positives can be a significant problem.

Examples are beginning to emerge from several efforts showing that progress has been made in characterizing orphan GPCRs. A first example is the identification of an orphan GPCR that functions as a calcitonin gene-related peptide (CGRP) receptor[45]. CGRP is a peptide of 37 amino acids, widely distributed in neurones, and functions as a potent vasodilator. It may be involved in migraine and has been implicated in non-insulin-dependent diabetes mellitus because it promotes resistance to insulin. An orphan GPCR EST was derived from a human synovium cDNA library[45]. Sequence analysis showed that the new GPCR has ~56% similarity to the human calcitonin receptor and was hence originally expected to be a new subtype of the calcitonin receptor. The message for this novel receptor was expressed predominantly in lung, which is known to be a relatively rich source of CGRP receptors. Following full-length cloning from a human lung library, the orphan receptor cDNA was stably expressed in HEK293 cells. Both radioligand binding using ^{125}[I]CGRP, as well as functional assays of CGRP-stimulated cAMP accumulation, demonstrated an appropriate pharmacological profile for the expressed receptor similar to that observed with endogenous CGRP receptors on human neuroblastoma cells. In addition to identifying the CGRP receptor, the reverse molecular pharmacology approach has also been used to identify other orphan receptors, such as the anaphylatoxin C3a receptor[46].

The examples given above are for receptors with significant homology to known GPCR superfamily members and their activating ligands proved to be known GPCR ligands. Will ligand fishing be successful in identifying novel endogenous ligands? Recently, two groups

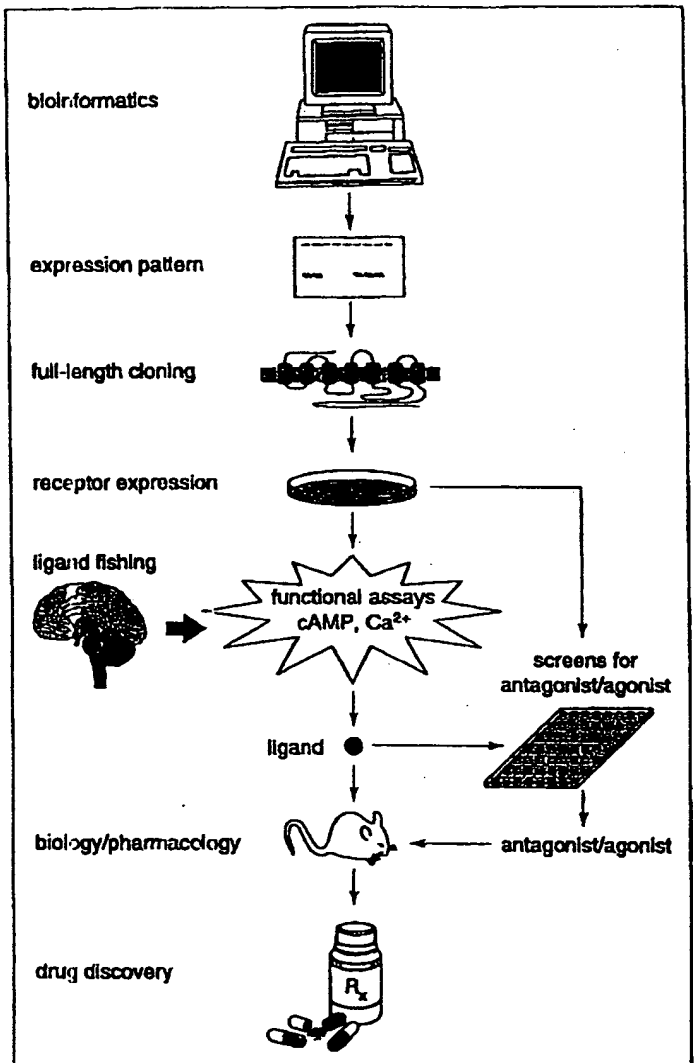

Fig. 3. Strategy for using orphan G protein-coupled receptors as targets for drug discovery.

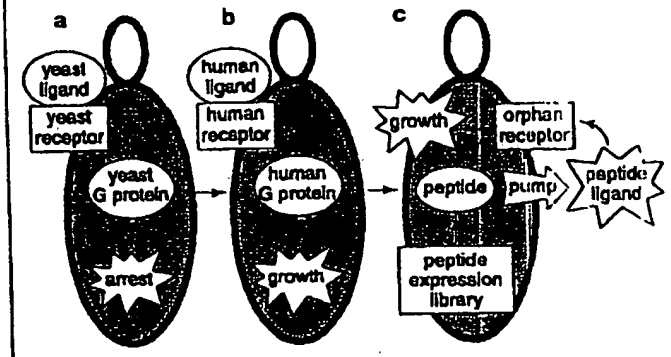

Fig. 4. Yeast-based screen for the identification of agonists for orphan G protein-coupled receptors (GPCRs). a: Normal, endogenous GPCR signalling in yeast (*Saccharomyces cerevisiae*). b: Substitution of a human GPCR and a human G_α subunit for yeast counterparts and modification of downstream signalling pathways such that agonist stimulation of the recombinant GPCR promotes growth. This yeast strain can be screened using biological extracts or compound libraries, or both. c: Yeast cells can be engineered to secrete small peptides from a random peptide library to identify autocrine surrogate, peptide agonists for recombinant orphan GPCRs. Modified from Ref. 49, with permission.

investigated an orphan opioid-like receptor, ORL1 (Refs 47 and 48). Both groups expressed the orphan GPCR in CHO cells and challenged the transfected cells with a series of opiate agonists, but without response. Both groups then used a similar ligand fishing approach. Taking crude extracts from rat brain[47] or porcine brain[48], they screened against the stably transfected cell lines using inhibition of adenylate cyclase activity as a functional assay. They were able to fractionate the brain extracts and identify the novel dynorphin-like ligand, which they called nociceptin[47] or orphanin FQ (Ref. 48). Thus, both teams successfully established a functional assay in transfected CHO cells that allowed the purification of a novel neuropeptide ligand that is 17 amino acids long for the orphan receptor. This work validates the ligand fishing approach for characterizing the function of orphan GPCRs.

Concluding remarks and future challenges

Although orphan GPCRs have been around for over ten years, very few companies have, until recently, been willing to risk their resources to explore opportunities among this category of receptors. However, the environment for the pharmaceutical industry has changed due to the confluence of several major technological advances. The conversion of gene sequences encoding GPCRs to drug targets is substantially aided by the development of combinatorial chemistry methods and miniaturized high-throughput screening techniques. The future challenge for drug discovery in this arena is to integrate these technologies innovatively and productively. One glimpse of the future comes from the field of functional genomics. The endogenous GPCR transduction system of the yeast, *Saccharomyces cerevisiae*, which is the pheromone pathway required for conjugation and mating, has been commandeered – through genetic engineering – to permit functional expression and coupling of human GPCRs and humanized G protein subunits to the endogenous signalling machinery[49–51] (Fig. 4). Further manipulations involve conversion of the normal yeast response to pheromone or activating ligand (growth arrest) to positive growth on selective media or to reporter gene expression. In addition, yeast cells have been engineered to express and secrete small peptides from a random peptide library that will permit the autocrine activation of heterologously expressed human GPCRs (Refs 49 and 51). This provides an elegant means of screening rapidly for surrogate peptide agonists that activate orphan receptors. This yeast system is, of course, not limited to autocrine ligand screening but can also be used in high-throughput mode to screen directly the fractions from biological extracts and the various chemical libraries as described above. A major advantage of the yeast system over the mammalian heterologous expression systems is its ease of use and its lack of endogenous GPCRs, which can confound ligand fishing expeditions in mammalian cells.

There is now tremendous pressure to be the first on the market with highly selective drugs that target therapeutic areas of unmet medical need and ideally have novel mechanisms of action. As a consequence, the pharmaceutical industry has recognized the power of genomics to provide it with new and unique drug targets. Genomics has responded with a plethora of novel proteins, included among them over 100 orphan GPCRs. Because of the proven link of GPCRs to a wide variety of diseases and the historical success of drugs that target GPCRs, we believe that these orphan receptors are, among the best targets of the genomic era to advance into the drug discovery process.

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CA_1A_2X-competitive inhibitors of farnesyltransferase as anti-cancer agents

Charles A. Omer and Nancy E. Kohl

For Ras oncoproteins to transform mammalian cells, they must be post-translationally farnesylated in a reaction catalysed by the enzyme farnesyl-protein transferase (FPTase). Inhibitors of FPTase have therefore been proposed as anti-cancer agents. In this review **Charles Omer and Nancy Kohl** discuss the development of FPTase inhibitors that are kinetically competitive with the protein substrate in the farnesylation reaction. These compounds are potent and selective inhibitors of the enzyme that block the tumourigenic phenotypes of *ras*-transformed cells and human tumour cells in cell culture and in animal models.

Since the identification of farnesyl-protein transferase (FPTase) activity in mammalian cells, there has been an intense effort to develop inhibitors of this housekeeping enzyme for use as potential, novel anti-cancer agents[1,2]. This idea stems from the fact that several of the proteins that regulate mammalian cell proliferation require a post-translational modification catalysed by this enzyme for biological activity. Efforts over the past eight years have yielded potent, cell-active inhibitors of FPTase that demonstrate anti-proliferative activity in cell culture and in rodent models of cancer.

The focus of the FPTase inhibitor (FTI) studies has been inhibition of the transforming activity of the Ras oncoproteins. Three *ras* genes, Ha-, N- and Ki-*ras*, encode four highly homologous, 21 kD proteins, Ha-, N-, Ki4A- and Ki4B-Ras (Ki4A- and Ki4B-Ras are encoded by splice variants of the Ki-*ras* gene)[3]. Ras functions to regulate the transduction of extracellular growth-promoting signals from membrane-bound receptor tyrosine kinases to intracellular growth-regulatory pathways. Typical of the low-molecular-weight G proteins, Ras is active when bound to GTP and inactive when bound to GDP. Cycling from the active to the inactive form is accomplished by the intrinsic GTPase activity of the protein. Mutations in Ras that abolish the GTPase activity result in constitutively active forms of the protein. Such oncogenically mutated forms of Ras, particularly Ki4B-Ras, are found in approximately 30% of many human cancers including 90% of pancreatic cancers and 50% of colon cancers[4,5].

Ras is synthesized as a biologically inactive, cytosolic protein that localizes to the inner surface of the plasma membrane where it acquires biological activity following a series of post-translational modifications (see Ref. 6 for review). The first and obligatory step in this series is the transfer of a 15-carbon isoprenoid, farnesyl, from farnesyl diphosphate (FPP) to the sulphur atom of the cysteine residue located four amino acids from the carboxyl terminus of the protein. This cysteine residue is part of the CA_1A_2X motif found in all FPTase protein substrates, where C is cysteine, A_1 and A_2 are usually aliphatic amino acids and X is usually serine, methionine, glutamine, alanine or cysteine. Following farnesylation, A_1A_2X is proteolytically cleaved and the now C-terminal farnesylcysteine is methylated. In the case of all of the Ras proteins except Ki4B-Ras, palmitate groups are then added to cysteine residues upstream of the farnesylated cysteine. The demonstration that farnesylation is essential for the transforming ability of the Ras oncoproteins[7–10] has spurred the development of inhibitors of the enzyme that catalyses this reaction, FPTase, as anti-cancer agents.

FPTase is a ubiquitously expressed, cytosolic enzyme comprised of two subunits, a 45 kDa α subunit and a 48 kDa β subunit[6]. Cross-linking studies have shown

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REVIEW ARTICLE

Orphan G-protein-coupled receptors: the next generation of drug targets?

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The pharmaceutical industry has readily embraced genomics to provide it with new targets for drug discovery. Large scale DNA sequencing has allowed the identification of a plethora of DNA sequences distantly related to known G protein-coupled receptors (GPCRs), a superfamily of receptors that have a proven history of being excellent therapeutic targets. In most cases the extent of sequence homology is insufficient to assign these 'orphan' receptors to a particular receptor subfamily. Consequently, reverse molecular pharmacological and functional genomic strategies are being employed to identify the activating ligands of the cloned receptors. Briefly, the reverse molecular pharmacological methodology includes cloning and expression of orphan GPCRs in mammalian cells and screening these cells for a functional response to cognate or surrogate agonists present in biological extract preparations, peptide libraries, and complex compound collections. The functional genomics approach involves the use of 'humanized yeast cells, where the yeast GPCR transduction system is engineered to permit functional expression and coupling of human GPCRs to the endogenous signalling machinery. Both systems provide an excellent platform for identifying novel receptor ligands. Once activating ligands are identified they can be used as pharmacological tools to explore receptor function and relationship to disease.

Keywords: Orphan; novel receptor; 7TM receptor; ligand fishing; novel peptide

Introduction

Rapid advances in DNA sequencing technologies have led to an exponential increase in the generation of genomic information. Such information holds enormous potential for drug discovery, allowing the identification of a diverse range of novel molecular targets. The challenge for the pharmaceutical industry in dealing with this wealth of new information is to identify the most promising candidates for further biological evaluation and to characterize their potential as drug targets as rapidly and efficiently as possible. This review will focus on one family of novel molecular targets, the family of orphan G-protein coupled receptors (GPCRs) and describe the strategies one can adopt to convert them into therapeutically relevant drug targets.

The superfamily of GPCRs is one of the largest families of genes yet identified. Over 800 members have been cloned to date from a wide range of species. The characteristic motif of this superfamily is the seven distinct hydrophobic regions, each 20–30 amino acids in length, generally regarded as the transmembrane domains of these integral membrane proteins. There is little conservation of amino acid sequence across the entire superfamily of receptors, but key sequence motifs can be found within phylogenetically related sub-families, and these motifs can be used to help classify new members.

Since the first cloning of GPCR cDNAs more than a decade ago, new genes have continued to emerge whose sequences place them firmly within the GPCR superfamily, but whose ligands remain to be identified. These 'orphan' receptors show low levels of homology with known GPCRs (typically less than 40%), too low to classify them with any confidence into a specific receptor subfamily. Many orphan receptors in fact show closer homology to each other than to known GPCRs, suggesting that they may represent new sub-families of receptors with distinct, possibly novel, ligands. These sub-families are distributed throughout the GPCR superfamily tree, suggesting that they will have a diverse range of functions.

What is the rationale for investing resources in characterizing orphan GPCRs? Simply stated, GPCRs have a proven history of being excellent drug targets. Several hundred drugs launched in the last three decades are directed at known GPCRs. Table 1 shows a representative snapshot of some well-established drugs and their corresponding receptors. It is clear that the therapeutic focus of these drugs spans a wide range of disorders from cardiovascular to gastro-intestinal to CNS and others.

Another example of the significance of GPCRs to drug discovery involves the increasing number of diseases associated with receptor gene mutations (Table 2). Some of the early examples identified involved mutations in receptors which caused gross changes in receptor function, leading to fairly rare but severe inherited disorders (e.g., mutations in vasopressin V_2 receptors associated with X-linked nephrogenic diabetes insipidus); (Birnbaumer, 1995). More recently, mutations have been identified that cause little or no apparent change in receptor function which lead merely to an increased propensity for developing a multifactorial disease (e.g. polymorphisms in the β_3-adrenoceptor associated with increased risk of insulin resistance); (Strosberg, 1997). As genotyping of disease populations becomes more comprehensive it is likely that more GPCR mutations or polymorphisms will be associated with disease states and hence provide additional potential targets for drug intervention. Since knowledge of gene function is not a pre-requisite for carrying out genotyping studies, it is just as likely that such mutations will be found in orphan receptors, which may provide clues to orphan receptor function in physiological and pathophysiological situations.

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Table 1 Drugs targeting GPCRs

GPCR	*Generic*	*GPCR*	*Generic*
Acetylcholine	Bethanechol Dicyclomine Ipratropium	Leukotriene	Pranlukast Zafirlukast
		Opioid	Buprenorphine Butorphanol Alfentanil Morphine
Adrenoceptor	Atenolol Clonidine Propranolol Terazosin Albuterol Carvedilol	Prostaglandin	Epoprostenol Misoprostol
Angiotensin II	Losartan Eprosartan	Somatostatin	Octreotide
		Serotonin	Sumatriptan Ritanserin Cisapride Trazodone Clozapine
Dopamine	Metoclopramine Ropinirole Haloperidol		
Histamine	Dimenhydrinate Terfenadine Cimetidine Ranitidine		

Table 2 Diseases associated with GPCR mutations

Receptor	*Disease*
Rhodospin	Retinitis pigmentosa
Thyroid stimulating hormone	Hyperfunctioning thyroid adenomas
Lutenizing hormone	Precocious puberty
Vasopressin V_2	X-linked nephrogenic diabetes
Calcium	Hyperparathyroidism, hypocalciuria, hypercalcemia
Parathyroid hormone	Short limbed dwarfism
β_3-Adrenoceptor	Obesity, NIDDM
Growth hormone releasing hormone	Dwarfism
Adrenocorticotropin	Glucocorticoid deficiency
Glucagon	Diabetes, hypertension

Much current research effort within the pharmaceutical industry today continues to focus on GPCRs, as they are justifiably perceived as attractive therapeutic targets. At SmithKline Beecham we have identified over 100 human orphan GPCRs that are distributed throughout the GPCR evolutionary tree. Some of these receptors are selectively expressed in a range of therapeutically relevant tissues, and it thus seems reasonable to speculate that they constitute a source of therapeutic targets with similar potential for drug discovery as seen with known GPCRs.

Reverse pharmacology approach

The overall strategy for characterizing orphan receptors has often been referred to as a 'reverse pharmacology' approach (Libert *et al.*, 1991) to distinguish it from more conventional drug discovery approaches. The conventional approach was historically initiated by the discovery of a biological activity for which the ligand responsible was identified and then used to characterize tissue pharmacology and physiological role. Subsequently, the ligand was used to clone its corresponding receptor for use as a drug target in high-throughput screening. The reverse approach starts with an orphan receptor of unknown function which is used as a 'hook' to fish out its ligand. The ligand is then used to explore the biological and patho-physiological role of the receptor. High throughput drug screening is initiated in parallel to develop tool compounds to help determine the therapeutic value of both agonists and antagonists to the receptor.

Screening strategy

Figure 1 illustrates the reverse pharmacology strategy adopted within SmithKline Beecham. The majority of our orphan receptors have been identified through extensive bioinformatic analysis of expressed sequence tag (EST) databases generated by mass random sequencing of cDNA libraries. The EST approach has previously proven to be a highly productive route for identifying novel genes (Adams *et al.*, 1992).

Since cDNAs identified by EST sequencing are often incomplete, the tissue expression pattern of the EST is analysed *via* Northern blot or RT–PCR to identify the tissue cDNA libraries which should be used to obtain a full length clone. More significantly, the expression pattern can determine whether a receptor is expressed in a normal or diseased tissue of interest as a therapeutic target. A highly selective tissue expression profile can also provide a clue to receptor function. For example, the expression pattern of the orphan receptor FC5 in rat brain as determined by *in situ* hybridization was recognized as resembling that of the Y_1 NPY receptor visualized by ligand autoradiography and hence led to the identification of FC5 as the Y_1 receptor. (Eva *et al.*, 1990). In parallel with obtaining a full length cDNA for the receptor, corresponding genomic clones can be obtained and their sequences from different individuals analysed to look for genetic markers that can be used to investigate possible associations with disease states.

Once a full length cDNA is available it can be expressed in mammalian cell lines for functional analysis. The choice of expression system is crucial to the success of ligand fishing: a system with a good history of GPCR expression which also contains a wide repertoire of G-proteins to allow functional coupling to down-stream effectors is of key importance. CHO (Chinese Hamster Ovary) or HEK (Human Embryonic Kidney) 293 cell lines are often the cells of choice, but as the success of receptor expression and coupling cannot be predicted, a variety of systems may have to be used. Alternative expression systems which can be used to explore different coupling mechanisms include xenopus oocytes, melanophores (Lerner, 1994) and engineered yeast systems (Broach & Thorner, 1996).

In the absence of a ligand to confirm receptor expression it is important to obtain some evidence that receptor transfections have been successful before embarking on the search for a ligand. Northern blotting of cell lines is probably the easiest test, but will only confirm that message for the receptor is present. To be confident that receptor protein is actually expressed one has to generate antibodies to the receptor or else attach an epitope tag to the receptor and assess protein expression *via* FACS analysis or Western blotting. A number of epitope tags have been successfully used to label GPCRs without affecting receptor function. Examples include FLAG and HA tags, for which antibodies are commercially available. (Guan *et al.*, 1992; Koller *et al.*, 1997).

The choice of functional assays used to screen for activating ligands is also critical to the success of ligand fishing. These should be as generic as possible to allow detection of a wide range of coupling mechanisms. Measurement of metabolic activation of cells expressing the orphan *via* the Cytosensor microphysiometer (McConnell *et al.*, 1992) is probably the most generic assay available, but is

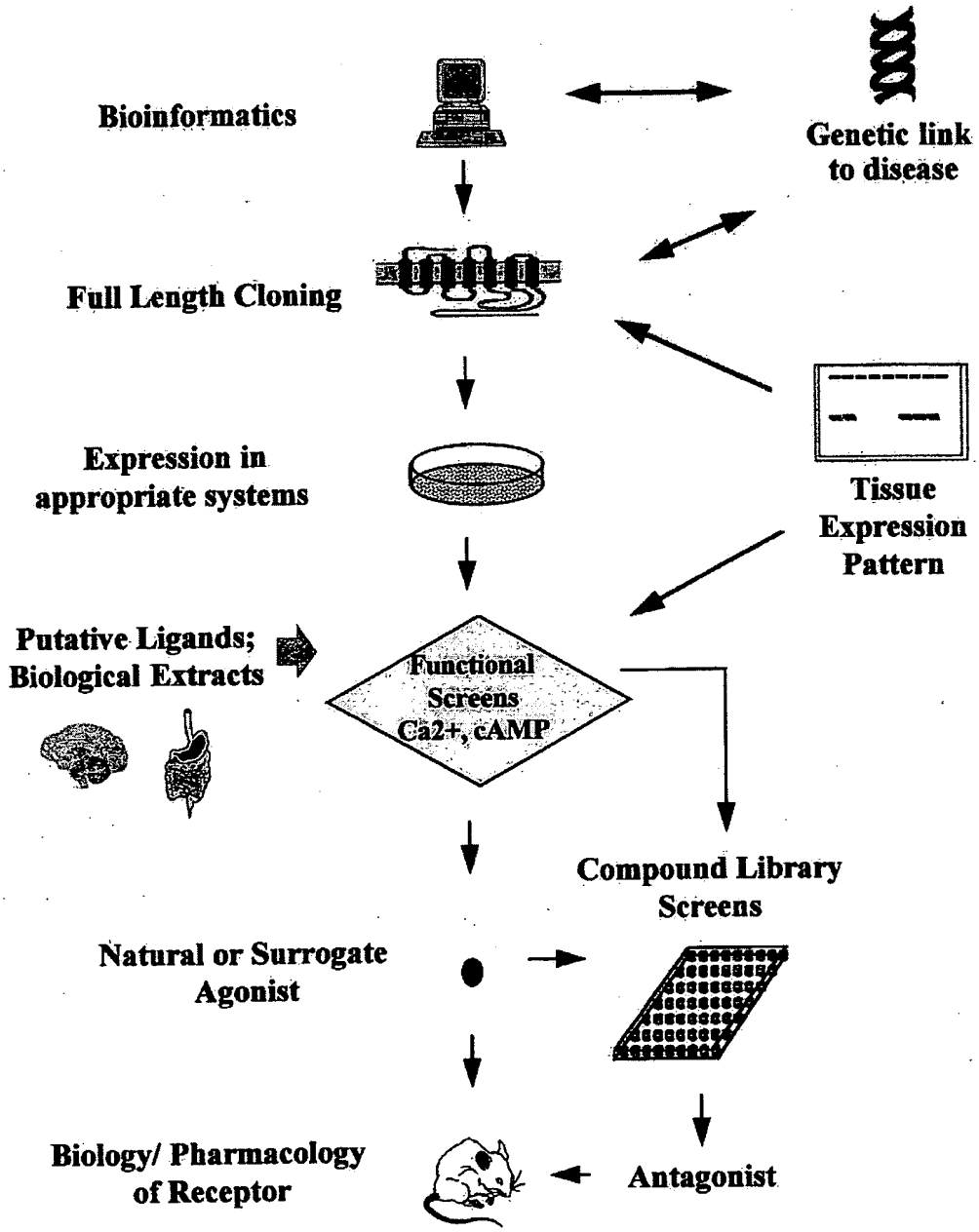

Figure 1 Reverse pharmacology approach to characterizing orphan GPCRs (modified from Stadel *et al.*, 1997).

hampered by its low screening throughput. Alternative assay systems in mammalian cells focus largely on measuring changes in intracellular cyclic AMP or Ca^{2+} levels, either directly using standard methods or *via* the use of reporter gene assays. It is becoming increasingly important to use high throughput systems to allow screening of large libraries of ligands, and assay technologies have evolved for each of these second messenger systems to allow high throughput readout in microtitre plate format. More recently, it has become possible to funnel heterologous GPCR signal transduction through a common pathway involving phospholipase C and Ca^{2+} mobilization by co-expression of the receptor with the promiscuous G-proteins $G_{\alpha 15/16}$ or with chimeric G_q-proteins such as G_{qi5} (Conklin *et al.*, 1996; Offermans & Simon, 1995). Although this approach may not work universally, the diversity of known GPCRs reported to successfully couple *via* these G-proteins suggests that it is a useful method to streamline screening for orphan receptor activation by focusing predominantly on one signal transduction system.

One factor which can complicate the use of heterologous expression systems for ligand fishing involves the presence of endogenous receptors in mammalian cell lines and in particular, clonal variation in the pattern of endogenous receptor expression in cells derived from the same parental cell line. Such variation has probably been responsible for the mis-

identification of a number of orphan receptors in the past. (eg, Cook *et al.*, 1992; Jazin *et al.*, 1993).

The ability to genetically delete endogenous GPCRs from yeast to generate a 'null' background is one of the major advantages in using yeast model systems for orphan receptor screening (Broach & Thorner, 1996). These systems rely on commandeering the endogenous yeast GPCR transduction system, the pheromone mating pathway, to allow coupling of transfected human GPCRs and humanized G-proteins to the endogenous signalling machinery. The manipulations involve conversion of the normal yeast response to pheromone activation (growth arrest) to positive growth on selective media, or to reporter gene expression. Such systems provide rapid, high throughput means of screening orphan receptors.

Once expression of the receptor has been achieved in mammalian or yeast systems and functional assays are in place, the search for activating ligands can begin. The receptors are screened initially against a bank of putative ligands, which includes known GPCR ligands as well as other naturally occurring bioactive molecules of unknown mechanism. The next step is to search for novel ligands by screening biological extracts obtained from tissues, biological fluids and cell supernatants. Another option is to screen peptide or compound libraries for 'surrogate' agonists that can be used as tool compounds. Yeast model systems here again provide unique advantages, in that they can be engineered to express and secrete small peptides from a random peptide library that will permit autocrine activation of heterologously expressed receptors, thus allowing a facile readout for detecting surrogate agonists of the receptor. Once an activating ligand has been found, whether natural or surrogate, it can then be used to explore the biology of the receptor *in vivo* or in tissue preparations, and can also be used to configure a high throughput screen to search for antagonists to support biological studies.

Characterization of orphan receptors

Examples are now emerging of the success of the reverse pharmacology approach in identifying orphan GPCRs. A first example is the identification of a calcitonin receptor-like orphan as a CGRP receptor. CGRP (calcitonin gene-related peptide) is a potent vasodilator widely distributed in central and peripheral neurones. It has also been implicated in migraine and non-insulin dependent diabetes. An EST derived from a human synovium library was used to clone a full length cDNA from human lung. Sequence motif analysis placed the receptor firmly within the secretin/VIP subfamily of receptors, with its closest homologue being the calcitonin receptor. However, the message for this novel receptor was expressed predominantly in lung, known to be a source of CGRP receptors. Following expression in HEK293 cells, the receptor was screened against putative ligands using a cyclic AMP stimulation assay (the primary signal transduction pathway for the secretin/VIP receptor family) and was shown to respond to CGRP with high potency (EC_{50} 0.9 nM) (Aiyer *et al.*, 1996).

The pharmacological profile of the receptor determined in the cyclic AMP assay and in radioligand binding assays was subsequently shown to be similar to that observed with endogenous CGRP receptors in human neuroblastoma cells, indicating that the orphan receptor cDNA encoded a CGRP receptor. In contrast, other workers who cloned this receptor failed to detect a CGRP response following expression of the cDNA in COS cells (Fluhmann *et al.*, 1995). The explanation for this discrepancy was initially thought to be due to lack of appropriate coupling machinery for the receptor in COS cells, as we also failed to detect a functional response with the receptor in this cell line. However, it has been shown more recently that accessory proteins appear to be necessary to allow this receptor to show CGRP responsiveness, so an alternative explanation may lie in differential expression of such accessory proteins in HEK293 vs COS cells (McLatchie *et al.*, 1998). Whatever the explanation, these findings illustrate that the choice of expression system is an important factor in identifying ligands for orphan receptors.

A second example of successful orphan receptor characterization was the identification of the receptor for the anaphylatoxin C3a (Ames *et al.*, 1996). The anaphylatoxins C3a, C4a and C5a are potent inflammatory mediators released during complement activation and they have been implicated in a number of inflammatory diseases. An EST derived from a human neutrophil cDNA library was used to clone an orphan GPCR from the same library. The receptor showed low homology to known GPCRs, with the best match being to the C5a receptor (37% nucleotide identity). However, Northern blot analysis indicated that its expression profile was distinct from that of the C5a receptor. Functional characterization of the C5a receptor was known to be problematic in cell lines such as HEK293 and CHO, but the receptor was known to be functionally active when expressed in RBL-2H3 cells (a rat basophilic cell line).

The orphan receptor was therefore expressed in RBL-2H3 cells and screened against putative ligands in a Ca^{2+} mobilization assay. It produced a robust Ca^{2+} mobilization response to C3a but not to C5a or other ligands. In parallel transfections in the same cell line the C5a receptor was shown to elicit a robust Ca^{2+} response to C5a but not to C3a. Radioligand binding studies using [^{125}I]-C3a confirmed that the orphan receptor bound C3a with a KD of 0.3 nM. These findings, together with the tissue distribution data, are consistent with this orphan encoding the human C3a receptor (Ames *et al.*, 1996).

CGRP and C3a constitute examples where known ligands have been paired with an orphan receptor. A third example involves the pairing of a novel ligand with an orphan receptor: the identification of a novel neuropeptide as an endogenous ligand for the receptor GPR10. The human orphan receptor GPR10 was first reported by Marchese *et al.* (1995), who cloned the receptor from genomic DNA using a PCR-based approach. Like the majority of orphan receptors, GPR10

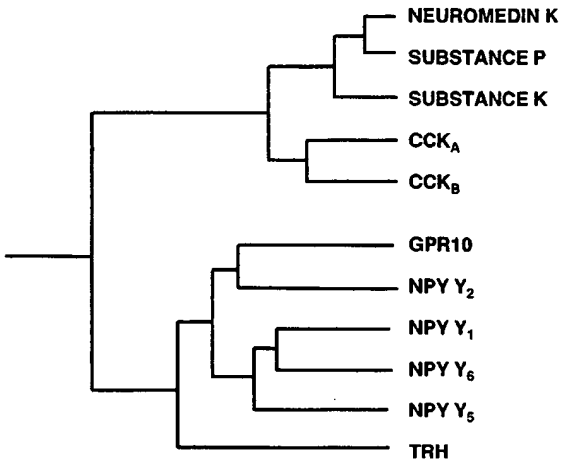

Figure 2 Phylogenetic tree for GPR10 and closely related receptors.

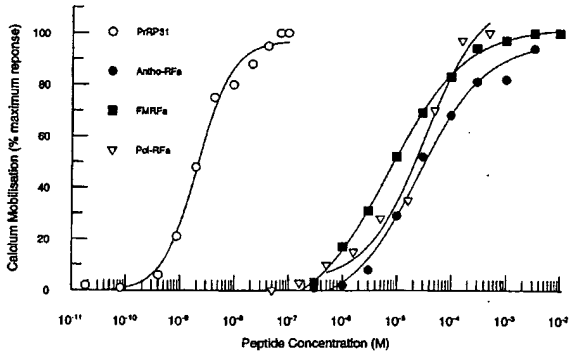

Figure 3 Ca^{2+}-mobilization responses to -RFamide peptides in HEK 293 cells stably expressing GPR10. EC_{50} values: PrRP31: 2 nM; *Antho*-RFa: 24 μM; FMRFa: 45 μM; *Pol*-RFa: 40 μM.

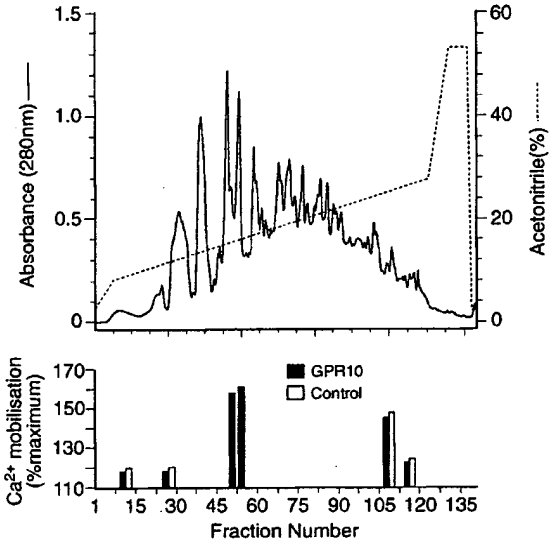

Figure 4 Stimulation of GPR10 by rp-hlpc fractions of crude peptide extracts of hypothalamus. Top panel, chromatogram. Acetonitrile gradient and absorbance of eluted materials indicated by dotted and solid lines respective. Bottom panel, Ca^{2+} responses in HEK293 cells stably expressing GPR10 or an unrelated orphan receptor (control). Peak Ca^{2+} responses were normalized to the maximum response induced by muscarine in the same cells. Note that a number of fractions induced similar responses in both cell lines, presumably acting through an endogenous receptor in HEK293 cells.

shows a low level of sequence homology with known GPCRs, although its closest match is to members of the NPY family of receptors (Figure 2). When expressed in HEK293 cells and screened against a bank of putative ligands, we found that the receptor failed to respond to NPY, but did produce robust Ca^{2+} mobilization responses to a number of short peptides found in invertebrates which are all members of the structurally related -RFamide peptide family (Figure 3). These peptides include FMRFamide, found in a variety of molluscan species, pQGRFamide (antho-RFamide) from *Anthopleura* and pQLLGGRFamide (pol-RFamide) from *Polyorchis* (Greenberg & Price, 1992).

The potency of these peptides at GPR10 was low (EC_{50} values in μM range – see Figure 3); moreover, the two known mammalian members of the -RFamide family, NPAF and NPFF, were inactive at GPR10. We hypothesized that the natural human ligand for GPR10 would be a novel member of the -RFamide family, perhaps a longer peptide which retained the characteristic -RFamide C-terminal motif. We therefore set out to purify the peptide from biological extracts of tissues.

High resolution reverse-phase HPLC fractions of a porcine hypothalamus extract were screened against GPR10 stably expressed in HEK293 cells and specific, robust Ca^{2+} mobilization responses were found in adjacent fractions (Figure 4). The activities appeared to be peptidic, as protease treatment of the fractions destroyed activity. In a parallel but unrelated effort, Hinuma *et al.* (1998) used very similar methods to also identify specific, peptidergic activity in hypothalamic extracts screened against GPR10 expressed in CHO cells, using arachidonic acid release as the functional assay. The activity was purified to homogeneity by further rounds of HPLC and ion exchange chromatography, and sequencing revealed a 31 amino acid peptide that, as predicted, contained a C-terminal -RFamide motif: SRAHQHS-MEIRTPDINPAWYAGRGIRPVG-RFamide. Hinuma *et al.* (1998) also found a second, overlapping peptide of 20 amino acids that appeared to be a truncated version of the longer peptide: TPDINPAWYAGRGIRPVG-RFamide. Cloning of the cDNA for the peptide revealed the existence of a prepro-peptide containing an N-terminal secretory signal, and confirmed the sequence of the peptide (Hinuma *et al.*, 1998). The authors found that the longer peptide stimulated prolactin release from pituitary cells *in vitro*, and termed the peptides PrRP31 and PrRP20 respectively. PrRP31 shows high potency at GPR10 (EC_{50} 2 nM – see Figure 3), contrasting the low potency of the peptides from invertebrates, and suggesting that this peptide is the endogenous mammalian ligand for GPR10. The role of the peptide *in vivo* is under evaluation.

GPR10 is the most recent example of the identification of a novel ligand for an orphan receptor using the reverse pharmacology approach. A second example involved the discovery of the orexins (orexin-A and orexin-B), two related peptides derived from the same precursor by proteolytic processing. Both peptides were purified from brain extracts by screening for Ca^{2+} mobilization responses against an orphan receptor expressed in HEK293 cells (Sakurai *et al.*, 1998). Orexin-B showed significantly lower potency than orexin-A at the orphan receptor, termed OX_1, which subsequently led to the discovery of a second orexin receptor (OX_2) at which both peptides were equi-potent. The orexin peptides are expressed in the hypothalamus and appear to be involved in the regulation of feeding (Sakurai *et al.*, 1998).

Novel non-peptide ligands have also been identified for orphan receptors, examples being anandamide and 2-arachidonyl glycerol as putative native ligands for cannabinoid receptors (Devane *et al.*, 1992; Stella *et al.*, 1997). As more orphan GPCRs are discovered and subjected to reverse pharmacology approaches, it seems likely that the number of novel ligands identified will increase. Moreover, as the biology of novel ligand/receptor pairs is evaluated and their association with disease states explored, we believe they will yield new drug targets that will allow us to treat a range of as yet unmet medical needs. As a consequence, orphan GPCRs may well provide the pharmaceutical industry with the next generation of drug targets (Stadel *et al.*, 1997).

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